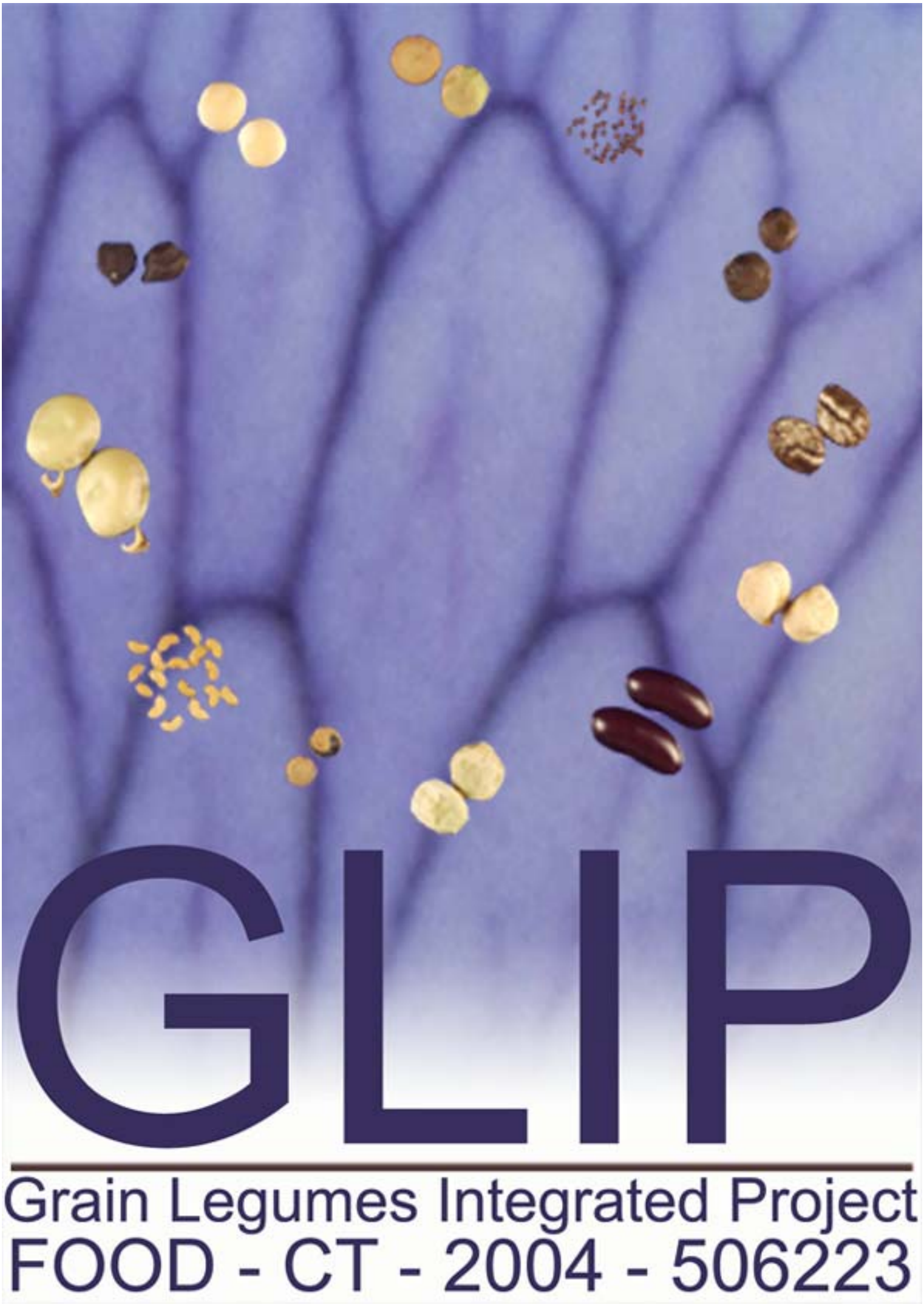
GLIP
Grain Legumes Integrated Project
FOOD - CT - 2004 - 506223

For

Yves Crozat and Markus Klenell

Two people who died while working within the Grain Legumes Integrated Project:

We had the privilege to have known them both; to have a sense of their humanity and their commitment to science. We therefore dedicate this last report from this project to their memory and to all who, like them, wish to contribute to humanity through science.

From all who worked on this project.

Summary

The Grain Legumes Integrated Project (GLIP) sought to achieve several general objectives.

i. To align research on grain legume crops in the EU and integrate this with related activities worldwide.
ii. To associate research on grain legume crops traits with biological studies in model systems.
iii. To develop genetic and genomic tools or the understanding and improvement of grain legumes.
iv. To disseminate knowledge and understanding generated by the project to all interested parties.

The EU continues to face the challenge of providing high quality protein for both animal and human consumption and currently imports about 70% of its plant protein, yet much of this could be derived from EU grown Grain Legumes. These are beneficial in the human diet and for animal feed. Furthermore, legume use in arable crop rotations reduces the need for fertiliser application and acts as a break-crop, reducing the need for pest and disease control while enhancing soil quality. The importance of this reduction in fertiliser dependence has become acutely interesting as something between 12% and 24% of total EU greenhouse gas production is a direct consequence of fertiliser application, mostly as a consequence of nitrous oxide production.

Grain legumes are underused by European farmers mainly because of yield inconsistency, so the Grain Legumes Integrated Project sought to mobilise and integrate European scientific research on grain legumes to underpin the redressing of this deficiency by:

i) Optimising parameters for legumes in feed quality and safety, while using legumes to develop healthy and sustainable agriculture.
ii) Investigating variation in grain legume seed composition and the factors affecting it.
iii) Developing new genetic, genomic, post-genomic and bioinformatic tools to improve and sustain grain legume seed production and quality.

To achieve these ends the project sought to integrate an ambitious combination of approaches, including biochemistry, plant & crop physiology, agronomy, plant genomics & breeding, and animal nutritional studies. Particular emphasis was placed upon the generation and use of state-of-the-art methodologies including systematic mutagenesis, genomics and bioinformatics, together with transcriptomics and metabolomics.

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The GLIP Partnership

The purpose of assembling the GLIP partnership was twofold: first we wanted to assemble technical and species-specific competences, and secondly we wanted to ensue maximal access to scientific advances within the European grain legume research community. These two intentions necessitated the establishment of large and complex consortium well suited to the structure of the EC instrument called an Integrated Project. The combination of the need of this community to integrate and develop common tools combined fortunately with the development of this structure.

During the course of the project the opportunity arose to extend the partnership to include 'Targeted Third Countries' and several laboratories were identified that had the potential to enhance the purpose of the consortium in the context of countries where grain legumes were of greater significance culturally, in agriculture and in the economy.

This combined to generate a consortium with 68 contractors at 80 locations where several locations had more than one participating laboratory, a scale that defies enumeration. Geographically, the distribution of participating laboratories is shown on the map below (Fig 1.)

Figure 1 Geographical distribution of GLIP participants.

● Coordinator, ● pre TTC Participants, ● post TTC Participants

A full participant list is given at the end of the document.

Project Structure.

The project was divided into Work Packages (WPs) in turn were grouped into Modules (Ms) of related activities. This structure facilitated scientific management and reporting and also allowed the management committees to weigh competing calls on limited resources. The Scientific Committee of the project comprised the WP and Module leaders while a smaller committee called the Governing Board that comprised the Module leaders and co-opted persons, permitted efficient decision making while retaining representation from the project as a whole. These WPs and Modules are listed below and provide the overall structure of this summary report.

The training WP provided resources for internal mobility allowing many exchanges between participating labs and in the final phase of the project enabled some targeted activities enhancing the output from the IP as a whole. Dissemination of information was ensured by networking with scientists and stakeholders inside and outside the GLIP consortium, both through individual contacts and via AEP, the European Association for Grain Legumes Research, and the Grain Legumes Technology Transfer Platform (GL-TTP) that was created within GLIP.

Module 1 Grain legumes and feed
Leader: A Jansman IDL
WP 1.1 Grain Legumes in Feed
Leader: A Jansman IDL
WP 1.2 Feed Processing and Nutritional Value
Leaders: K Hasenkopf & U. Knauf, Fraunhofer

Module 2 Economic and environmental impact
Leader: ES Jensen TUD / (Risø)
WP2.1 Lower Input Farming
Leader: ES Jensen TUD
WP2.2 Economic and Environmental Analysis
Leader: T Nemecek ART

Module 3 Seed composition and quality
Leader: M Stitt MPG
WP3.1 Systems Approaches to Seed Composition
Leader: R Thompson INRA Dijon
WP3.2 Novel Approaches to Alter Seed Composition
Leader: C Domoney JIC

Module 4 Crop functioning and seed quality
Leader: N Ellis JIC
WP4.1 Abiotic Stress
Leader: M Crespi CNRS Gif
WP4.2 Biotic Stress
Leader: D Rubiales CSIC Córdoba
WP4.3 Plant Architecture
Leaders: C Rameau INRA Versailles
& F Madueño CSIC Valencia
WP4.4 Carbon/Nitrogen Allocation and Seed Quality
Leader: C Salon INRA Dijon

Module 5 Genetic and genomics tools
Leader: Jean Dénarié INRA Toulouse
WP5.1 Sequencing
Leader: G Oldroyd JIC
WP5.2 Mutagenesis and reverse genetics
Leaders: A Konorosi & P Ratet CNRS Gif
WP5.3 Expression Profiling
Leader: H Kuester Uni. Bielefeld
WP5.4 Crop & Comparative Genomics
Leader: G Kiss IG ABC

Module 6 Bioinformatics
Leader: K Mayer MIPS
WP6.1 Bioinformatics

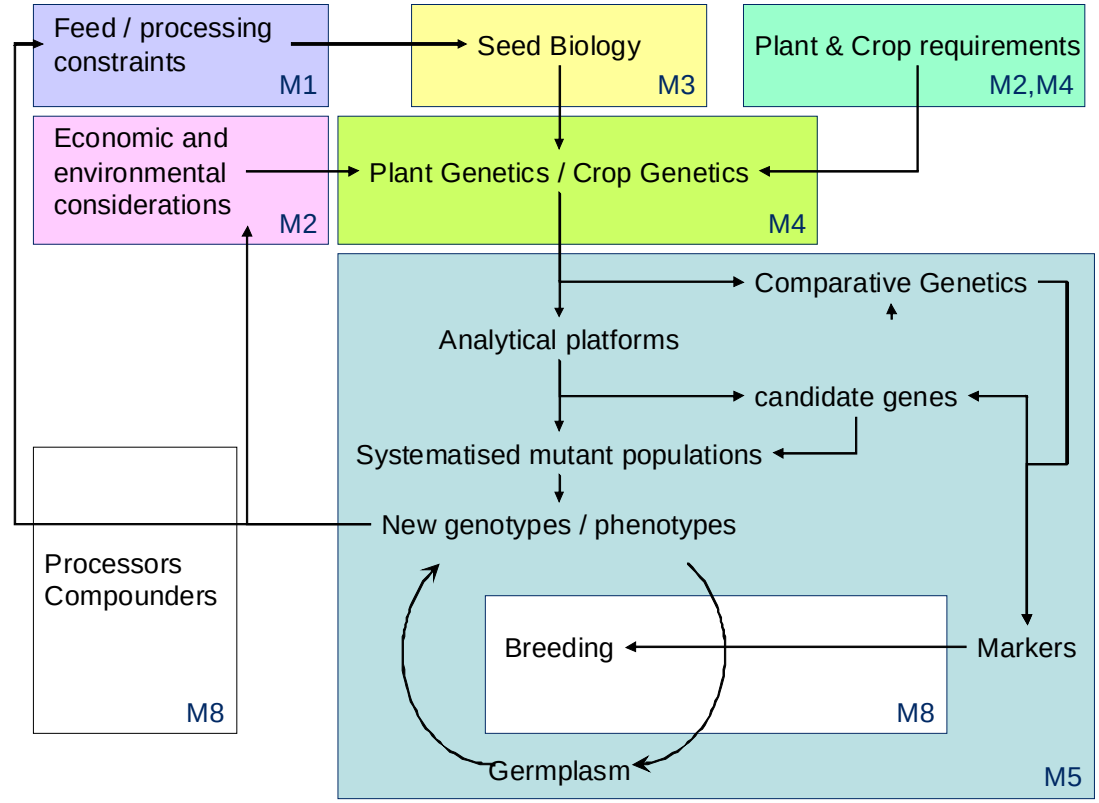
Module 7 Coordination and training
Leader: T Bisseling Uni. Wageningen
WP7.1 Coordination
Leader: N Ellis JIC
WP7.2 Training
Leader: T Bisseling Uni. Wageningen

Module 8 Dissemination and transfer
Leader: F Muel UNIP
WP8.1 Dissemination of Knowledge
Leader: A Schneider AEP
WP8.2 Transfer and Exploitation of Results
Leader: C Golstein GL-TTP

Integration

The purpose of assembling a partnership with species-specific and technical competences was to promote the connectivity between research demands, activities and outputs. This can be illustrated diagrammatically:

Figure 2 Integrative research.



In large part the objectives of the project were to harness new genomic and post-genomic tools for both crop genetics and improvement, but to guide this process with input from agronomic, economic or end-use considerations.

Module and Work Package highlights and overviews

WP 1.1 Grain Legumes in Feed

The European feed industry relies, to a large extent, on the import of protein sources such as soybean meal from South America for its requirement for protein-rich ingredients. From an environmental and sustainability point of view an increase in grain legume production in Europe and an increase of its use in animal feed could contribute to a more sustainable form of animal production. The use of feed ingredients in animal feeds is based on their availability, their nutritional value relative to the price level and to other functional characteristics of the feed ingredients (improvement in feed processing characteristics, positive effects on the palatability of the feed or improvement of animal or intestinal health).

This work package has focussed on the improvement in knowledge on the nutritional value of European legume seeds, pea in particular and on the evaluation of functional characteristics of European grain legumes to support gut health in pigs and poultry. For this purpose tools have been developed to assess the effects of grain legumes on nutritional value and to determine the effects grain legumes on microbial activity in different parts of the digestive tract of pigs and poultry by measuring the non enzymatically digested fractions, which could potentially be used as substrate for growth of intestinal microflora. The intestinal microflora in farm animals has important effects on gut health characteristics. In addition, *in vitro* studies were made on the binding characteristics of grain legume fractions towards pathogenic microbial species in the gut.

A schematic presentation of the core activities within the work package “GL in feed” is indicated in Figure 3.

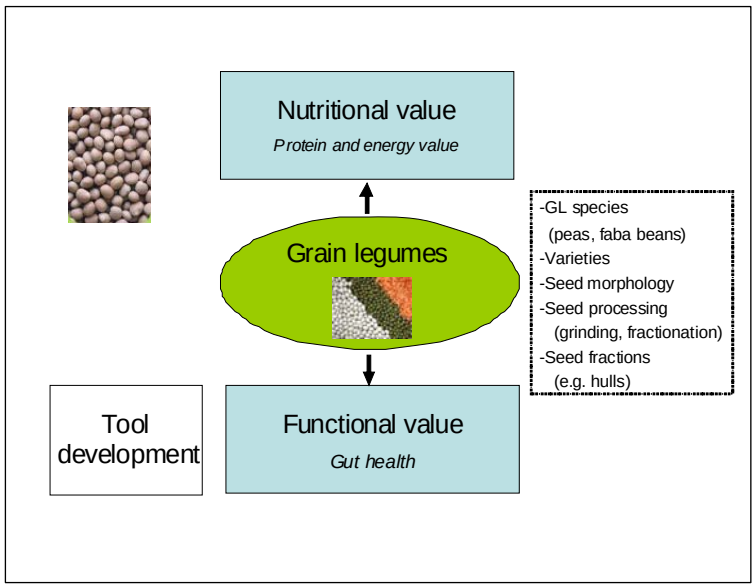


Figure 3. Schematic presentation of the research focus in “GL in feed” in GLIP.

In summary, the aims of the work package were to:

1. Optimise the nutritional value of grain legumes as ingredients in animal feed and determine characteristics of grain legumes that support intestinal health and function of pigs and poultry.
2. Develop an *in vitro* assay for the determination of nutrient digestibility and degradation kinetics in order to predict nutritive value of grain legumes and the substrate availability for microflora in different parts of the gastro-intestinal tract of pigs.
3. Evaluate pea cultivars with distinct chemical composition and cotyledon cell structure and morphology to optimise nutritional value and health benefits as ingredients in animal feed.
4. Determine the effects and functional properties of processed legumes and/or fractions on the intestinal health of young piglets.

Enzymatic digestion in the small intestine and fermentation in the distal part of intestinal tract of piglets was simulated by *in vitro* techniques. Enzymatic digestion is mimicked by *in vitro* incubations with pepsin and pancreatic enzymes. Measurements of hindgut fermentation were based on the cumulative gas production of feed ingredients by micro flora and enabled quantification of fermentation kinetics. The *in vitro* digestibility and fermentation kinetics of several feed ingredients and grain legumes, as well as complete diets including these ingredients, were determined. The assays can be used to quantify the effect of grain legumes on nutritional and functional properties in diets of weaned piglets and growing finisher pigs.

In addition, *in vitro* adhesion tests were performed evaluating the effects of peas and faba beans and fractions of these legumes towards relevant pathogens (*E. coli* and *Salmonella enterica*) in a microtitration plate-based adhesion assay. The results (Figure 4) showed that fibre rich fractions of pea could potentially adhere (pathogenic) bacteria in the gut of pigs, which could contribute to a lower colonization of pathogens in the GI tract and a lower susceptibility of the gut for disease under challenged conditions. The effects of pea and faba bean fractions on the consequences of an oral challenge with an enteropathogenic *Escherichia coli* (ETEC) were evaluated in post weaning piglets. The results showed that legume seeds are suitable protein sources in diets for young piglets.

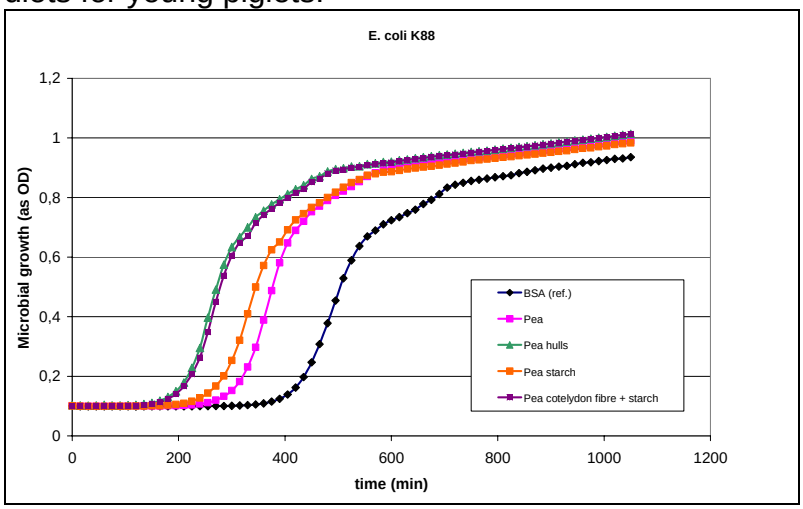


Figure 4.
Effects of various pea fractions on the in vitro binding of a pathogenic *E. coli*, based on bacterial growth curves. A large binding capacity is represented by an early start of the growth curve in the graph.

The GLIP project has generated new and practically relevant information on the nutritional value of grain legumes in feeds for pigs and its factors of variation, namely the sensitivity of seeds to the grinding process and consequences on protein and amino acid digestibility. Secondly, interesting results were found on the capacity of European legume seeds to contribute to improving and supporting gut health of farm animals, e.g. by binding pathogenic bacteria in the intestinal tract resulting in a lower risk of intestinal colonisation of these bacterial species. The validation of these results in animal studies and their application under more practical conditions requires further attention.

WP 1.2 Feed Processing and Nutritional Value

The aim of this workpackage was to examine the potential of processing crude grain legumes to improve the nutritional value of legume-based products. These results will help to make legumes an interesting raw material for high value feed and supplement the efforts of new breeding strategies. Processes that were examined were hydrothermal treatment, germination and air-classification. The efficacy was evaluated *in-vitro*, in digestibility trials and in feeding trials with salmon and piglets.

An efficient method for increasing the nutritional value was air-classification. Dehulled, finely ground pea or faba bean meal is thereby separated in a stream of air into a coarser and a finer fraction. The smaller protein particles accumulate in the fine fraction, the starch granules in the coarse fraction [Figure 5].

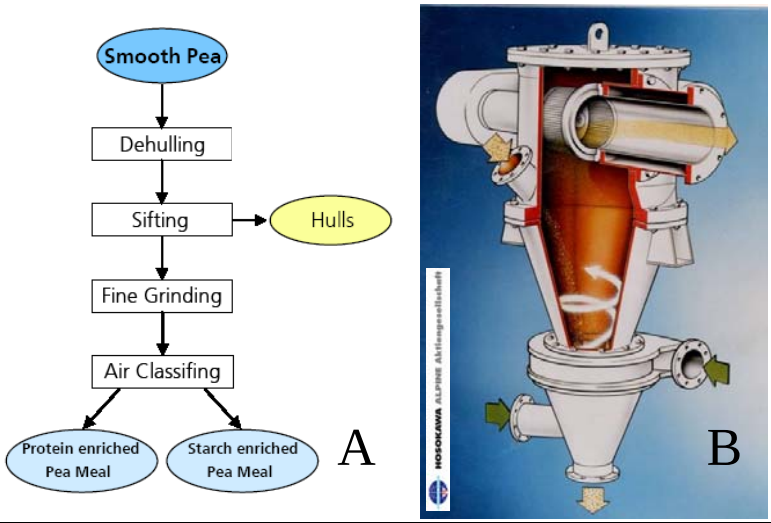


Figure 5 Air-classification of peas
A. Procedure B. Air-classifier Turboplex ATP 50 (Hosokawa Alpine)

It was possible – depending on the settings - to produce pea protein concentrates with more than 60 % protein and faba bean protein concentrates up to 70 % protein. At the same time, fat, minerals, and several critical compounds like phytic acid, trypsin inhibitors and α-galactosides were accumulated.

These protein concentrates were tested in digestibility trials with Atlantic Salmon. The inclusion level of the legume products was 30 % and the protein content of the feed was adjusted to an equal level. The apparent protein digestibilities of the protein concentrates were for all materials higher than the digestibility of the respective meal from dehulled seed [Figure 6]. Especially for pea the effect was impressive. Lupin meal also showed very good results even without protein-enrichment.

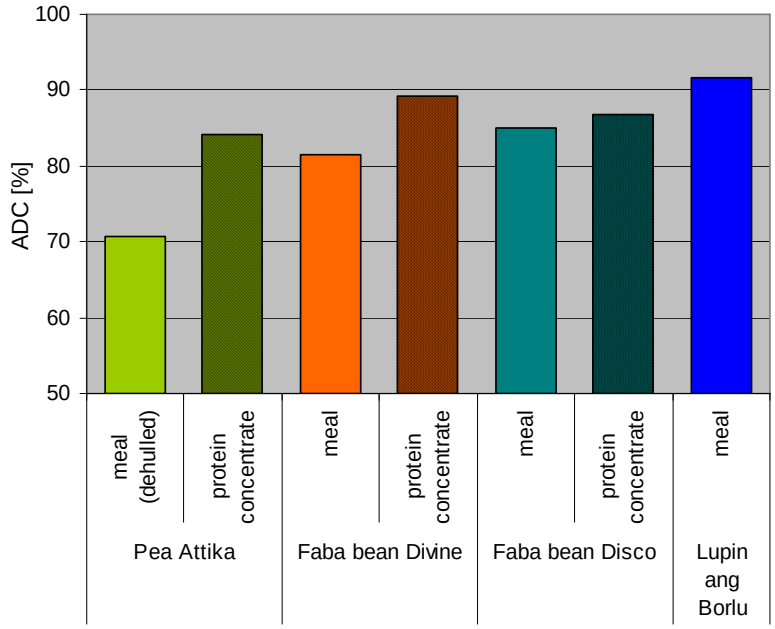


Figure 6 Partial protein apparent digestibility coefficients (ADC) of faba bean, pea and lupin products

In a growth trial, the inclusion of 10 and 20% legume meal showed no significant differences on the growth of salmon compared to a control diet with fish meal. Higher inclusion rates were not tested within the project.

Positive results for faba bean concentrates were also obtained in a growth experiment with just weaned piglets. The results clearly indicated that growth performance with diets containing a low tannin faba bean protein concentrate was similar to the performance obtained with a diet containing a protein concentrate from soybeans, the latter being a benchmark product in these kinds of diets. Also important was the observation that the occurrence of digestive disturbances was not increased with diets containing the faba bean protein.

Not only nutritive but also technological impacts of the incorporation of high amounts of pea protein concentrates from air classification in fish feed formulations were tested. Fish feed is usually produced in a cooking extrusion process followed by vacuum coating with oil.
Replacing 50 % of fish meal with legume meal compared to a formulation containing only fish meal and wheat starch showed – at the same settings - a significant decrease in expansion of the pellets during the cooking extrusion process. Consequently, less oil could be added during the coating. At the same time, the pellet hardness and durability were reduced.
The rate of expansion could be influenced by process parameter like temperature, screw speed, and water content during cooking extrusion [Figure 7]. An improvement of the pellet properties could be achieved by identifying accurate, modified settings during cooking extrusion and vacuum coating (pressure, oil quantity, temperature). With these settings, the incorporation of

pea protein meal at a 50 % replacement rate was possible without major negative effects on the pellet quality.

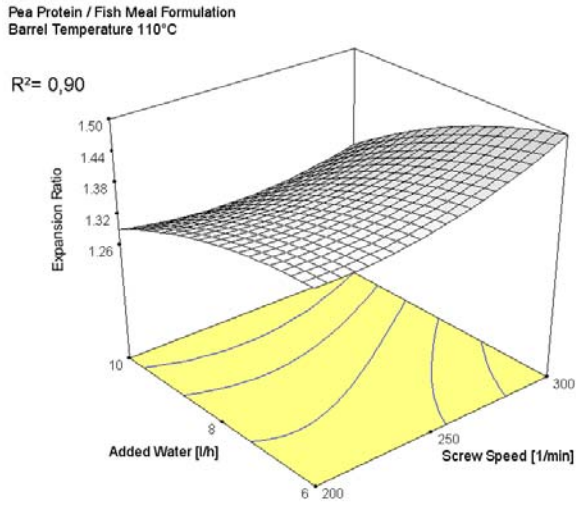


Figure 7 Impact of water content and screw speed on the radial expansion of fish feed pellets from a modified formula containing pea protein

These trials showed that it is possible to produce legume-rich fish feed pellets. The process tends to become more sensitive, particularly when starch content is further reduced or additional oil is added. Therefore, a careful adjustment of process parameters becomes necessary when higher rates of plant proteins are used in the formulation.

In summary, the results of workpackages 1.2 proved that air classification is an very interesting methodology for increasing the nutritional value of legumes. The protein concentrates can replace significant amounts of fish meal and therefore support a sustainable aquaculture. Another application in feed can be the replacement of soy proteins concentrates as it could be seen in the piglet trials. Air-classification is a relatively cheap technology for protein concentration, as no drying steps are necessary. The starch-enriched by-fractions can be used for fattening feed, for technological purposes (e.g. plastic films, alcohol manufacture) and as ingredients for the food industry.

Module 2 Economic and environmental impact

Module 2 was designed to understand agronomic approaches that maximise the benefits of grain legumes in crop rotations, while minimising any potential for adverse effect. Furthermore grain legumes are currently 'under used' so we need to understand this in the economic and environmental context in which grain legumes are produced and used. This module was "*a multidisciplinary approach*" by contributing agroecological understanding "from crop physiology and agronomy" in their economic context, with the intention that this can inform effective strategies in plant breeding and agriculture.

The lower input farming workpackage focused on the new cropping technologies for grain legumes in European arable rotations and an improved understanding of the beneficial and the potential risks associated with grain legumes. We found that growing grain legumes with other species in an intercrop can reduce disease and parasitic weed infection and thus potentially the requirement for pesticides. The most serious disease in pea is pea root rot and it was found that the disease epidemic is mainly due to primary (soil) infection, and not a spread in the crop from secondary infections. The work also included selection and test in farmers field of Phaseolus bean lines with high and low phosphorous use efficiency. Such lines are relevant for elucidating the mechanisms behind high P use efficiency in low P soils. This knowledge is highly relevant for stabilizing the yield of grain legumes.

Nitrogen derived from atmospheric N_2 fixation is a key benefit from grain legumes. The spatial variability of pea was assessed in 10 ha fields and the work revealed a very high degree of variability in N_2 fixation, which we could not correlate to basic soil parameters as total N, P, pH or texture. The work also pointed to weaknesses in the well-know natural ^{15}N isotopic method to determine N_2 fixation, since there was also a high variability at the short-scale (c. 30 cm). The fixed N benefits the legume and the subsequent crop via deposition of N in the roots and other residues. The research resulted in the first estimates of N rhizodeposition from pea in the field and showed that that the total proportion of plant N in roots and root residues may be as high as 30%. Furthermore the method for determining rhizodeposition of N was significantly improved. Analysis of the soil N dynamics in cropping systems with grain legumes (Toulouse, France and Taastrup, Denmark) showed that more N is present in the soil after legumes than after cereals and that this N can be protected from being lost by growing a catch crop. After the catch crop is incorporated in the soil, this N can contribute to succeeding crop N supply. The research showed that it is important to carry out experiments with crop sequences to understand the beneficial effect of grain legumes in a cropping system. The collaboration between French and Danish research groups in this context was essential to obtain an understanding of the dynamics and effects in very different European environments.

The results of workpackage Economic and Environmental Analysis show that the European deficit in protein concentrates could be reduced by increased grain legume production in Europe, but the situation varies greatly between

countries. Peas as the main species are well suited for pig compound feed. A feedstuff model yielded the surprising result that the European potential is about 4 times higher than the current use (1 million tonnes).This discrepancy is explained mainly by the limited availability of peas on the market, despite the fact that the profitability of crop rotations with grain legumes is at least as high as that of common crop rotations. Furthermore it was revealed that the main economic value of peas is its energy rather than its protein content, especially for monogastric animals.

The life cycle assessment (LCA) studies of animal production systems showed that replacing soya bean meal by peas and faba beans in animal feed reduced transport impacts significantly, but did not produce an overall environmental advantage. The studies also showed that e.g. on-farm feedstuff production reduces the environmental burdens of animal production.

The differences between pork produced with soya bean-based feed and pea-based feed were small, as were the differences between human meals, which included pork chops produced from these two feeds. The fully vegetarian alternative had clearly lower overall environmental impacts, but needed a lot of energy for processing, showing a great need for optimisation.

The economic and environmental analysis in the GLIP-project showed that there is potential for peas on the feedstuff market, but that a simple replacement of soya bean meal by peas in animal feedstuff does not guarantee a more environment-friendly animal production or human food sector.

WP2.1 Lower Input Farming

Grain legumes have the unique ability to fix atmospheric dinitrogen and consequently need no N-fertilization (less fossil energy use and CO_2 / N_2O emission), they diversify crop rotations and increase soil and plant health. If the European societies want to optimize these benefits for agriculture and the environment, the crop yields for legumes needs to be stabilized to encourage increased use by European farmers and an improved understanding of basic agroecological processes are required. The overall goals in WP2.1 were to evaluate innovative grain legume management strategies for European cropping systems, and to consider the crop and rotational health of farming systems, minimize energy use and nutrient losses to the environment.

Intercropping for reduced pesticide use. We determined the mechanisms by which intercropping (the simultaneous cultivation of more than one species in the same field) can reduce plant diseases (*Ascochyta* blight*)* and parasitic weeds in grain legumes and how these effects may be managed for better crop yield and grain quality. The research showed that the reduction in Ascochyta blight from intercropping, e.g. with barley or wheat was greatest when the epidemic was severe. The reduction was caused by the physical barrier of the intercropped species to the splash dispersal of spores and/or an intercrop mediated change in micro climate, but also the in the intercropped species/cultivar and sowing patterns influenced the degree of disease reduction. Intercropping also reduced the parasitic weed broomrape. The effect on yield of the intercrop mediated disease/weed reduction needs to be further evaluated.

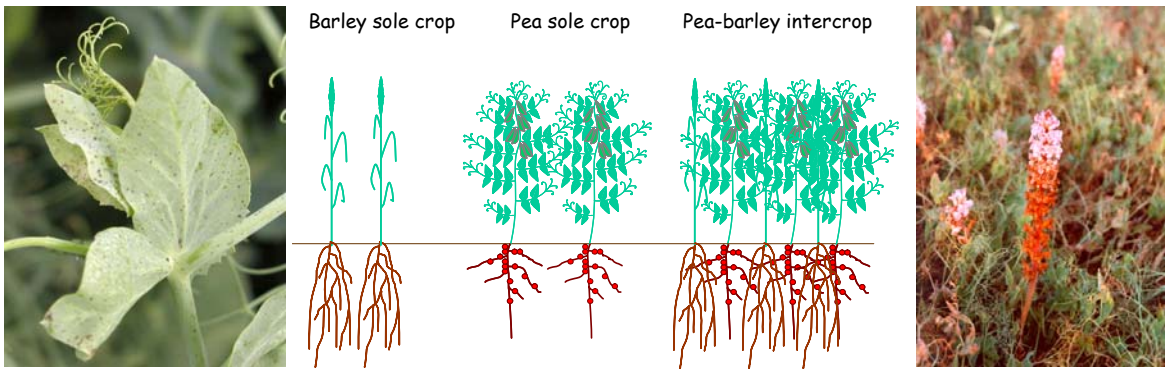


Figure 8
Ascochyta blight on pea | Principle of intercropping | Broomrape on pea

Spatio-temporal dynamics of pea root rot (Aphanomyces) Pea root rot is a serious pathogen in pea. When a field is infested by this soil-borne pathogen, it may take many years before peas can be grown again in the same field. We studied the dynamics of the pathogen at the field scale during a crop cycle to develop improved preventive measures. The main result is that the disease spread to neighboring plants only within a distance of max 15 cm. This indicates that field epidemics are mainly related to primary (soil) infections, and that a low sowing rate can reduce the spread of the disease in the crop.

Figure 9
Aphanomyces inoculum source

Genotypes for efficient use of phosphorus in low P soils

Low phosphorous availability has a great negative impact on grain legume yield. We selected and assessed Phaseolus bean genotypes for contrasting phosphorus use efficiency in controlled and field conditions to understand why some genotypes fix N_2 and reach greater yields under low availability of P. The lines showed significant differences in response to low P soils and the potential mechanisms were studies, such as nodulation and phosphatase activity causing increased solubilization of P. The lines can be used to identify low P soils and soils where P is a limiting factor for N_2 fixation in bean.

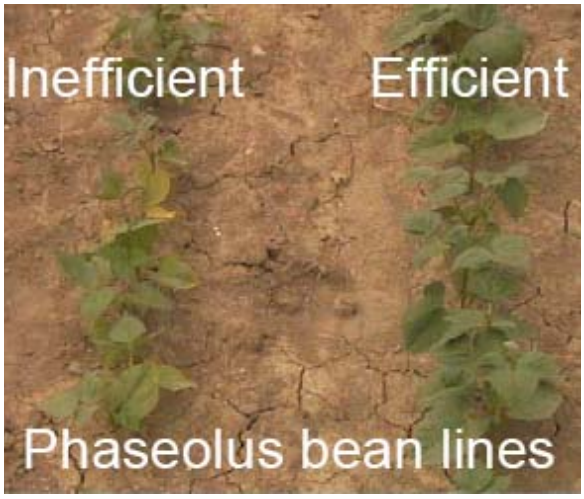


Figure 10
Phosphorous use efficiency differences

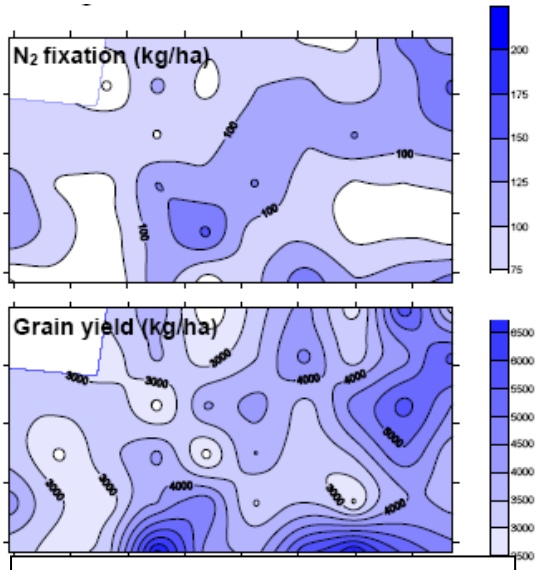


Figure 11
Field scale variability

Spatial variability in N_2 fixation at the field scale

Grain legume yields are known be more unstable between years and sites than cereal yields. However, within field spatial variability in yield and N_2 fixation and the extent to which this variability is correlated with soil parameters are unknown. We studied the variability within 10 ha fields and found a very high variability, which could not be correlated to the soil parameters available (total N, P, pH, texture, etc.). The research further showed that the N_2 fixation is highly variable within short distances (down to 30 cm) and also pointined to weaknesses in the ^{15}N isotopic method used to estimate N_2 fixation.

Rhizodeposition of N from grain legumes

Grain legumes can contribute to building soil N reserves and the N-supply of subsequent crops via deposition of N from roots. We developed new methods for quantification of this pool of nitrogen, quantified it in the field and greenhouse. In green-house grown pea the N in rhizodeposition was found to vary between 12 and 30% of the total plant N, whereas it constituted 30-55% in the field. Genetic or environmental factors such as N supply did not seem to influence the rhizodeposition.

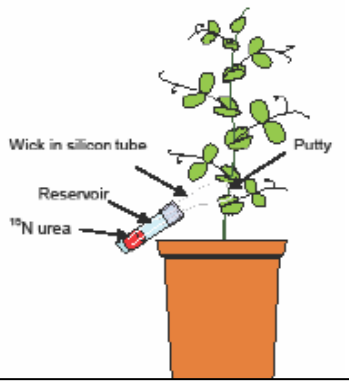


Figure 12
Cottonwick labelling of pea for determination of N rhizodeposition

Soil N dynamics in crop rotations with grain legumes

The greater mineral N often found in the soil after grain legumes can benefit subsequent crops eventually via a catch crop which is grown in autumn and subsequently incorporated before establishment of the succeeding crop. We determined the effects of grain legume residues, their quality and catch cropping on soil nitrogen dynamics, nitrate leaching and the succeeding crop nitrogen accumulation in northern and southern European cropping systems with grain legumes. To be able to design better cropping system for the future, we need model tools which can simulate the effects of grain legumes on agronomic performance and the environment. We improved the French STICS model for simulation of grain legume N_2 fixation and nitrogen dynamics including nitrogen leaching in crop rotations with grain legumes. The results confirmed that soil cultivated with grain a legume contains higher amounts of nitrate at harvest than soil cultivated with cereals, However, growing catch crop after the grain legumes minimized the nitrate leaching and conserved the extra N after grain legumes for subsequent crops. It was observed that the catch cropping effect on subsequent crops was greater than the effect of the main precrop, e.g. legume or cereal, and the composition of the catch crop greatly influences the succeeding crop effect in at least the two main crops following grain legumes.

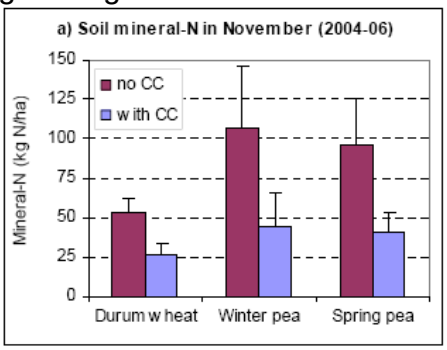


Figure 13
Soil mineral N with and without catch crop in a cropping system with GL in Toulouse

Cropping system experiment and soil sampling in Taastrup, Denmark

WP2.2 Economic and Environmental Analysis

The objectives of WP 2.2 were twofold:

1) to assess the economic and environmental impacts of grain legumes in animal feed and human nutrition,
2) to identify constraints to the increase of grain legume use.

Two tools were used to analyse these questions:

(i) economic feedstuff modelling (linear programming), allowing the calculation of optimal feedstuff formulas based on the composition and price of raw materials and the cost of transport
(ii) life cycle assessment (LCA), addressing the environmental impacts of products and production systems throughout the whole life cycle.

Our results show that the European deficit in protein concentrates could be reduced by increased grain legume production in Europe, but the situation varies greatly between countries. Peas as the main species are well suited for pig compound feed and also for poultry and dairy cows, as long as the production intensity is not too high.

The feedstuff model yielded surprising results: the potential use of peas in the seven countries examined (Belgium, the Czech Republic, Denmark, Germany, the Netherlands, Spain and the UK) was estimated at 4.1 million tonnes, whereas current production is only 1.0 million tonnes! In Spain (Figure 14) the model estimates that pea use could be increased even by a factor 7!

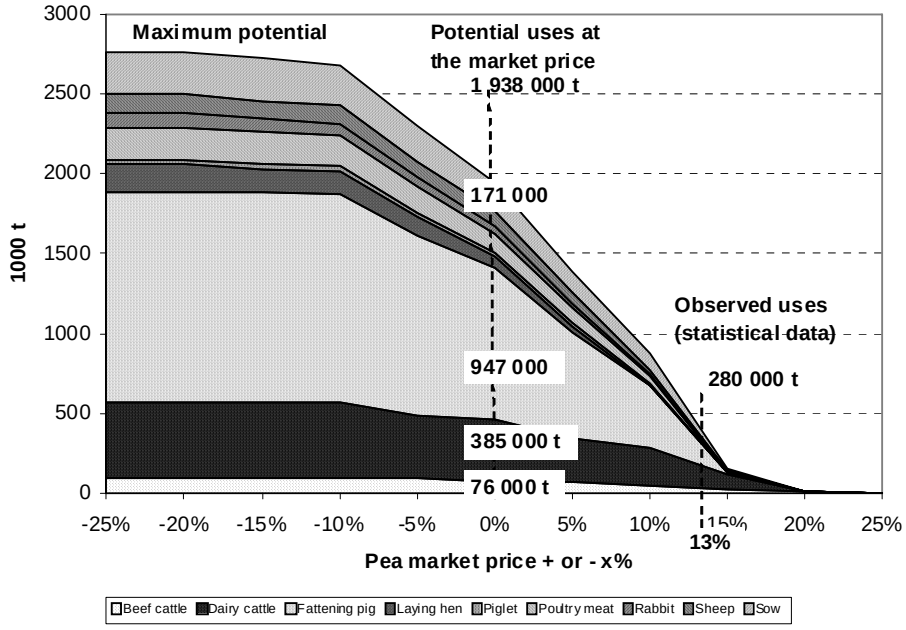


Figure 14. Assessment of potential pea uses by animals according to the variation of its market price in Spain in 2004.

This discrepancy is explained mainly by the limited availability of peas on the market. But why do we have this discrepancy between the demand for peas

and their supply on the market? The profitability of crop rotations with grain legumes is at least as high as that of common crop rotations. Further research is needed to better understand the functioning of the pulse feed market.

In contrast with many people's belief, the main economic value of peas was in its energy rather than its protein content, especially for monogastric animals. Furthermore, the feed evaluation method used for ration formulation strongly affects the inclusion of peas: the net energy system appears to justify potentially higher pea use than the digestible or the metabolised energy systems. The feedstuff model showed that peas are not an essential part of compound feed formulas, since they can be substituted easily by other raw materials.

The life cycle assessment (LCA) studies of animal production systems (Figure 15) showed that replacing soya bean meal by peas and faba beans in animal feed reduced transport impacts significantly, but did not produce an overall environmental advantage. That depended much more on the composition of the feed. Since the nutritional properties are different, there is not a direct 1:1 replacement of soya bean meal by peas. The nutritional properties of the replacing ingredients are different, and therefore the whole feed formula must be changed. As the feedstuff optimisation model has purely economic goals (minimum price); it is not surprising that the environmental impacts are not necessarily reduced. By including environmental criteria in the optimisation models, an overall improvement could probably be achieved. This would require information on environmental impacts for all feedstuffs, but this is not available currently. The studies also showed that other options exist to reduce the environmental burdens of animal production, with regional or on-farm feedstuff production and improved manure management being the most important ones.

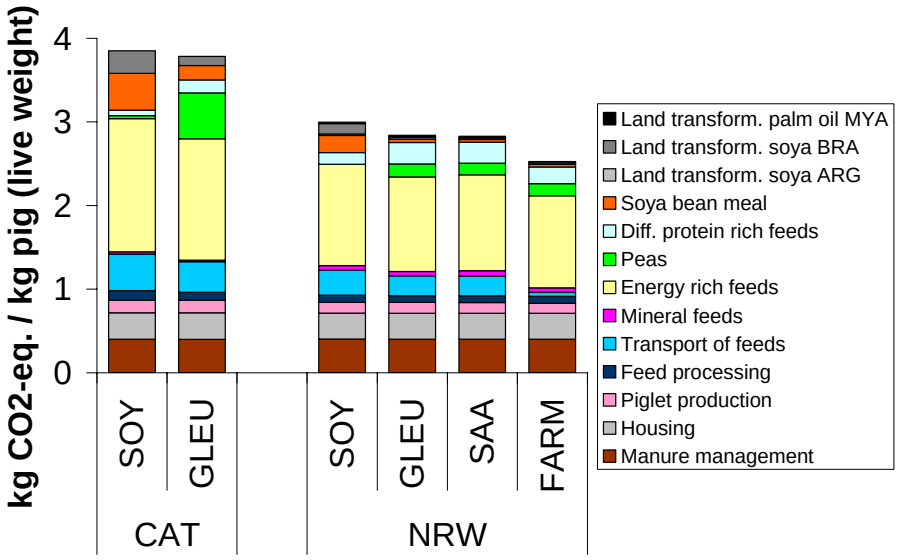


Figure 15. Global warming potential per kg pork produced in Catalonia (CAT) and North-Rhine Westphalia (NRW) with soya bean meal from overseas (SOY), European grain legumes (GLEU), synthetic amino acids (SAA), or on-farm feed production (FARM).

The differences between pork produced with soya bean-based feed and pea-based feed were small, as were the differences between human meals, which included pork chops produced from these two feeds. The meal with partial replacement (10%) of meat by pea protein had a slightly higher environmental impact. In such a meal much more meat would have to be replaced in order to achieve a substantial reduction of the environmental burdens. The fully vegetarian alternative had clearly lower overall environmental impacts, but needed a lot of energy for processing, showing a great need for optimisation. Environmental impacts can be decreased further by reducing food waste at all stages (increasing efficiency of food use) and by improvements in food ingredient production in agriculture.

The economic and environmental analysis in the GLIP-project showed that there is potential for peas on the feedstuff market, but that a simple replacement of soya bean meal by peas in animal feedstuff does not guarantee a more environment-friendly animal production or human food sector. Further efforts are necessary to give grain legumes their place in a sustainable agriculture.

Module 3 Seed composition and quality

For most legumes, the seed represents the part of the plant that is harvested for use as feed or food. Their seeds are a rich source of protein, reflecting the ability of legumes to perform fixation in their symbiotic nodules in association with *Rhizobia* species. Legume seeds also provide calories, normally in the form of starch. Legume seeds therefore form an important component of the human diet, especially in societies where meat is an expensive commodity or, for reasons of conviction, there is a high proportion of vegetarians. Legumes are also an important component of animal feed.

Improvement of seed composition and quality is an important goal of legume breeding. On the one hand, it would be advantageous to increase the overall protein content. Additionally, it would be desirable to modify the amino acid composition of the protein to increase the levels of certain essential amino acids. Another important breeding goal is to decrease the level of unwanted compounds in legumes, including allergens. For example, many seed storage proteins have allergenic properties. Plant breeding depends upon identifying or creating appropriate genetic diversity, and then analysing it to identify desired traits. This was previously a very empirical process. It is becoming increasingly important to support this process by more detailed analyses of the genetic structure of populations, and a molecular and physiological analysis of the biological processes that underlie the desired traits. In this module, two complementary and interlocking work packages explored novel strategies to use genomics to improve legume composition and quality.

Work package 3.1 used a systems approach to analyse seed composition and quality. It used natural diversity as a source of genetic variation. Marker-assisted breeding in a crop plant was combined with the use of modern analytic tools in genomics and metabolomics, to analyse the biochemical and molecular mechanisms that underlie traits that are relevant for nutrition. Genetically-well characterised pea lines with improved protein levels or composition were taken as the starting point. They were subjected to a broad analysis to gain insights into the underlying causes of the changes, to assess any negative impacts these changes have, and to screen for patterns and clusters in metabolites and transcripts that could allow nutrition-related traits to be monitored by proxy.

Work package 3.2 used genomics approaches to identify key genes that may regulate seed composition. The ultimate aim was to identify key regulatory genes that control pea seed development and composition. However, genomics approaches are used more efficiently in model species, where there are more resources and technical possibilities. While many of the basic signalling processes regulating nitrogen allocation and use in plants are likely to have widely common features, we can also expect some species-specific regulation mechanisms. This work package therefore carried out a detailed analysis of events during seed filing in the premier model plant, *Arabidopsis thaliana*, and in a model legume, *Medicago truncatula*, to discover possible candidate genes. These were supplemented by some focused studies to test the effect of altering the expression of a small number of candidate genes in pea.

Many seed proteins are stored in specialized structures, the protein storage vacuoles (PSVs). Within WP3.1, pea seed PSVs were purified, and their constituent proteins analysed. A total of 252 proteins were identified, many of which are novel. Some of these are candidates for determining the final seed protein content in seeds.

Several of the Module 3 studies linked activities in both work packages. For example, a pea genotype was identified that lacked a potential allergen (PA2), but contained more protein in its seeds (JI 1345, marked with an asterisk in Figure 16). A lack of PA2 is due to a defect in the gene(s) encoding this protein, where most, or all, of the gene is missing (Figure 16A, central panel). The mutant line was crossed with a conventional cultivar (Birte), so that the effects of the mutation could be examined in a standard background.

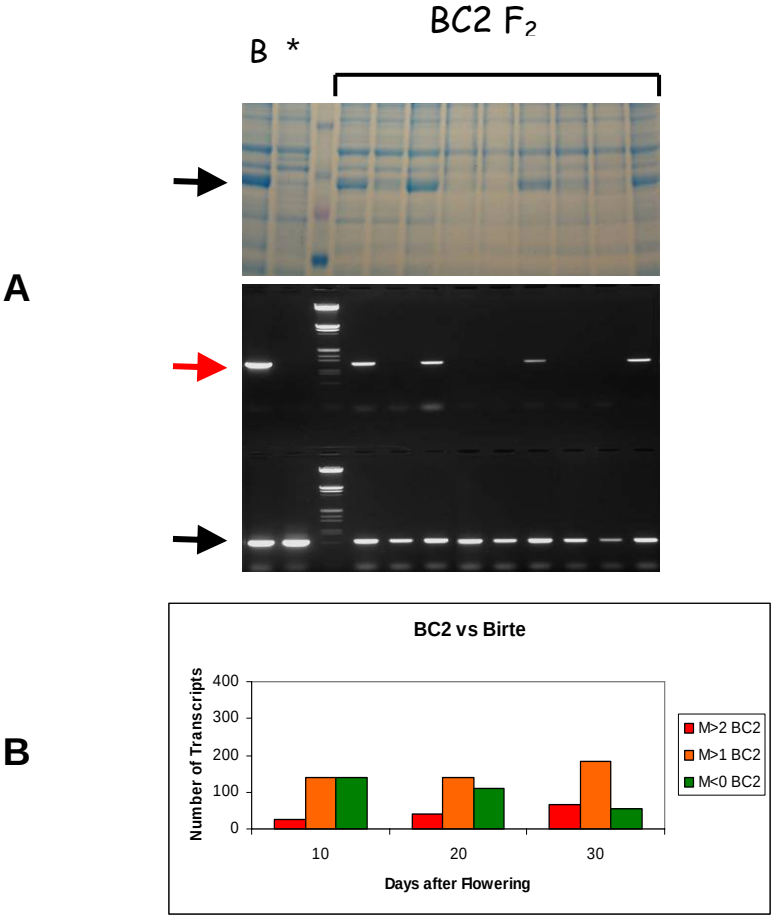
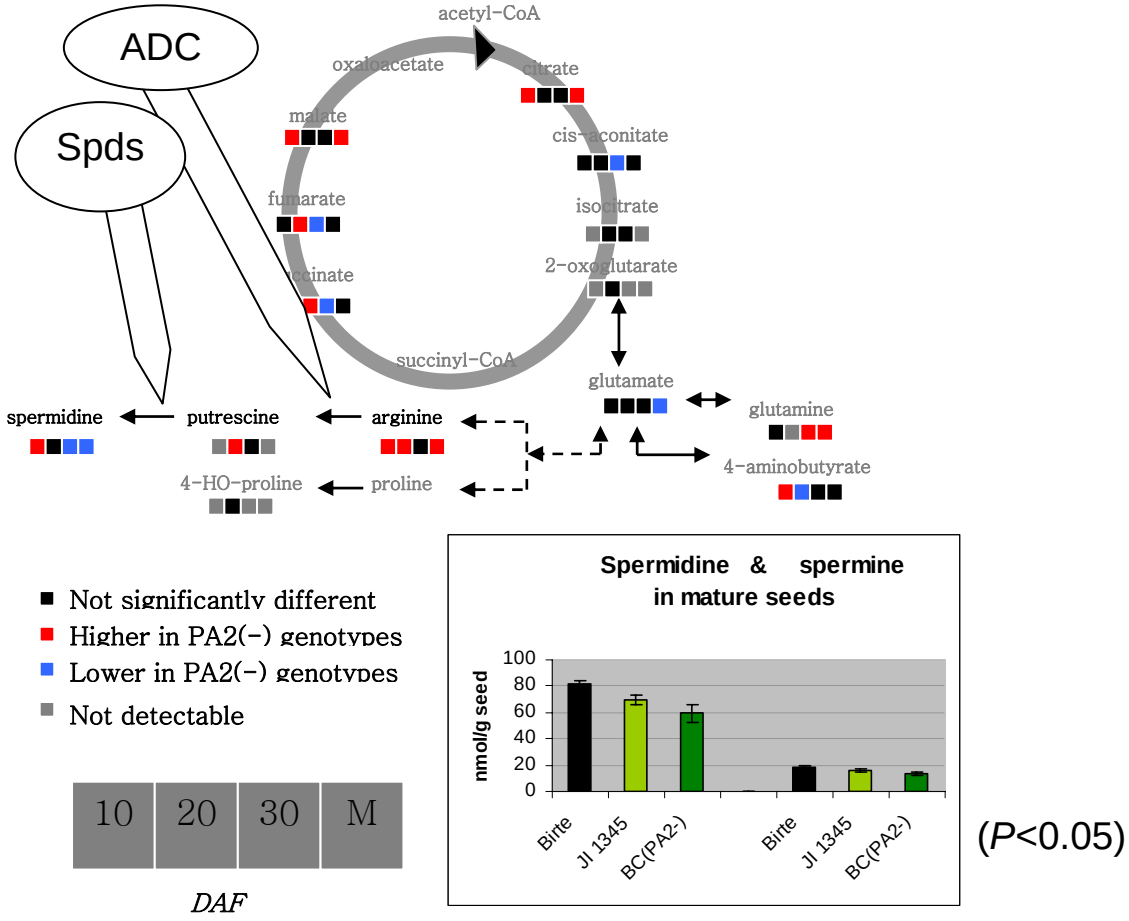


Figure 16. *A: The inheritance of PA2 is shown (arrows) for seed protein (top panel, black arrow) and DNA (mid panel, red arrow) for parent (B, *) and back-cross (BC) F2 lines. A control DNA screen is shown in the lowest panel (black arrow). Track B = Birte, * = parent line lacking PA2. B: The numbers of genes whose expression changes during seed development in the BC mutant at 10, 20 and 30 days of pod development relative to the cv. Birte. Red, orange and green are highly up-regulated, moderately up-regulated and down-regulated, respectively, in the mutant line lacking PA2.*

The amounts of protein and metabolites (small compounds) were compared in the back-cross lines lacking PA2 (Figure 16A) and cv. Birte. Overall changes in gene expression were monitored in order to account for these changes. Figure 16B summarises the numbers of genes whose expression is significantly changed in the mutant, compared with cv. Birte. Significant changes in the amounts of amino acids (building blocks for proteins) and other small nitrogen-containing metabolites, called polyamines, were apparent in the mutant. Figure 17 shows some of these differences, where red blocks indicate a significantly higher content of a particular compound in the lines lacking PA2, at a defined stage of seed development (10, 20, 30 days after flowering or in mature seeds).



($P<0.05$)

Figure 17. *The changes that occur during seed development in pea lines lacking PA2. Coloured blocks indicate whether higher or lower amounts of a particular compound are present in the mutant lines, at four stages of seed development (days after flowering, DAF, or mature seeds, M). The positions at which two key enzymes operate are indicated with large open arrows (arginine decarboxylase, ADC, and spermidine synthase, Spds). The bar chart shows the amounts of the polyamines, spermidine and spermine, present in mature seeds of three pea lines, two of which lack PA2 (JI 1345, BC(PA2-).*

The activities of two enzymes that contribute to polyamine content, arginine decarboxylase (ADC) and spermidine synthase (Spds) (Figure 17) were found to be lower in the mutant lines, and this explains the significantly lower amounts of spermidine and spermine in these lines at maturity (Figure 17). None of

these changes appear to negatively affect early plant growth in mutant lines, although this needs to be examined rigorously under field conditions. In another part of the project (Module 1), the overall properties of the seed proteins were tested. The missing protein (PA2) can negatively affect some industrial uses of protein, and so the mutant line shows advantages for these uses.

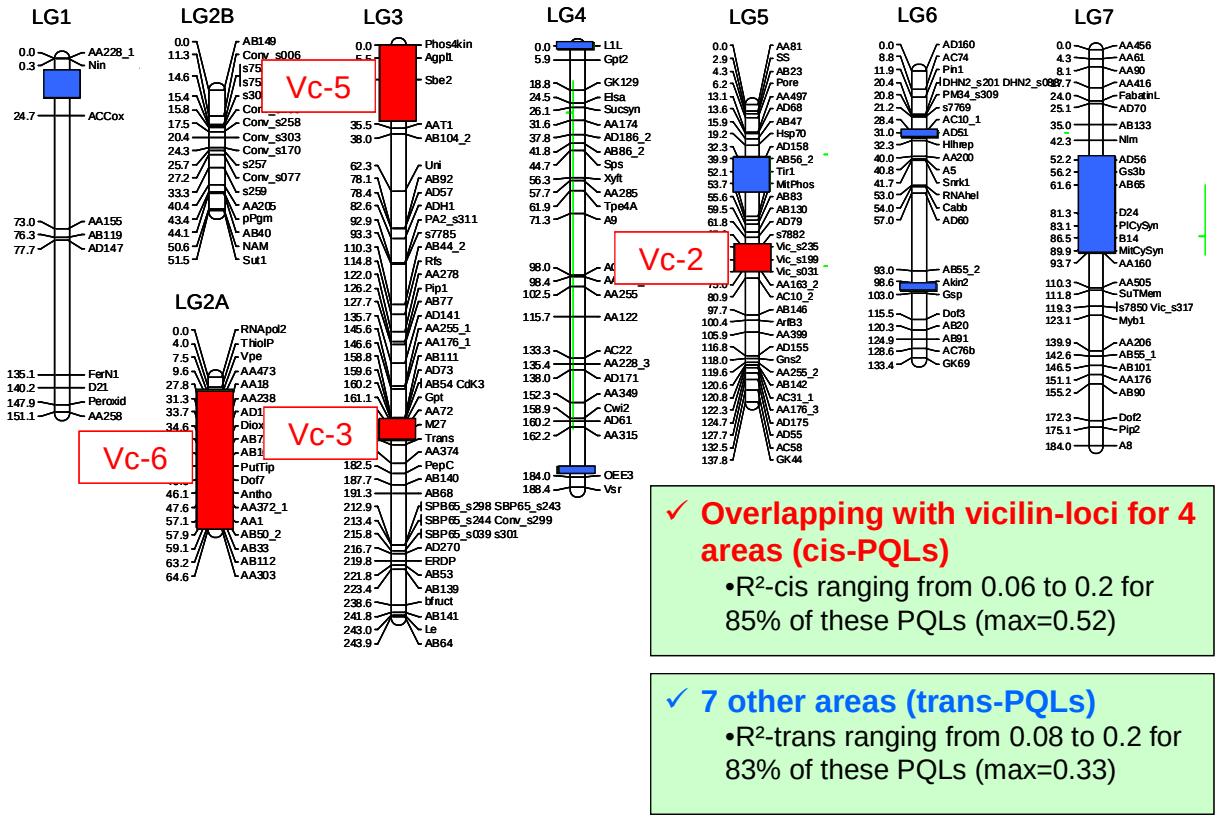


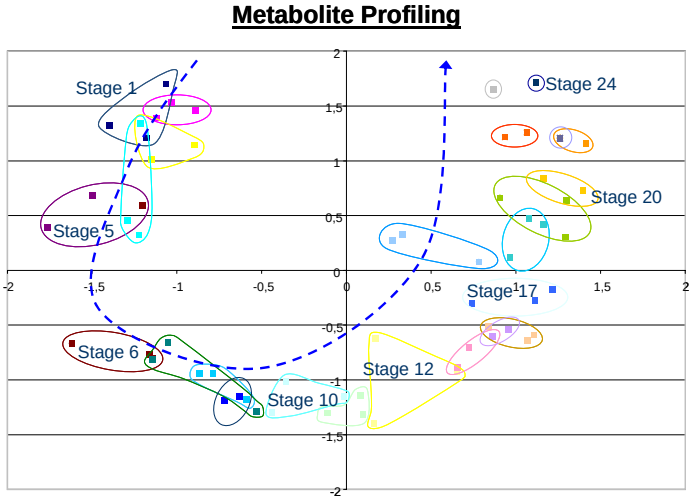
Figure 18. *The relative position of genes controlling the quantity of the pea seed protein, vicilin, on the seven linkage groups of pea*

The amounts of some pea seed proteins were shown to be controlled by several genes, and these were found to be widely distributed on the genetic map. For example, the content of vicilin, a major seed storage protein class, was found to be controlled by at least 11 genes (Figure 18). Some of the controlling genes co-segregated with genes that encode the different individual vicilin proteins (red zones in Figure 18). This may mean that the genes encoding individual vicilins also control their relative amount, as has been shown to be the case for other seed proteins. Additionally, there are genes that control the relative amount of vicilins, that lie in regions of the map distant from the encoding genes (blue zones in Figure 18).

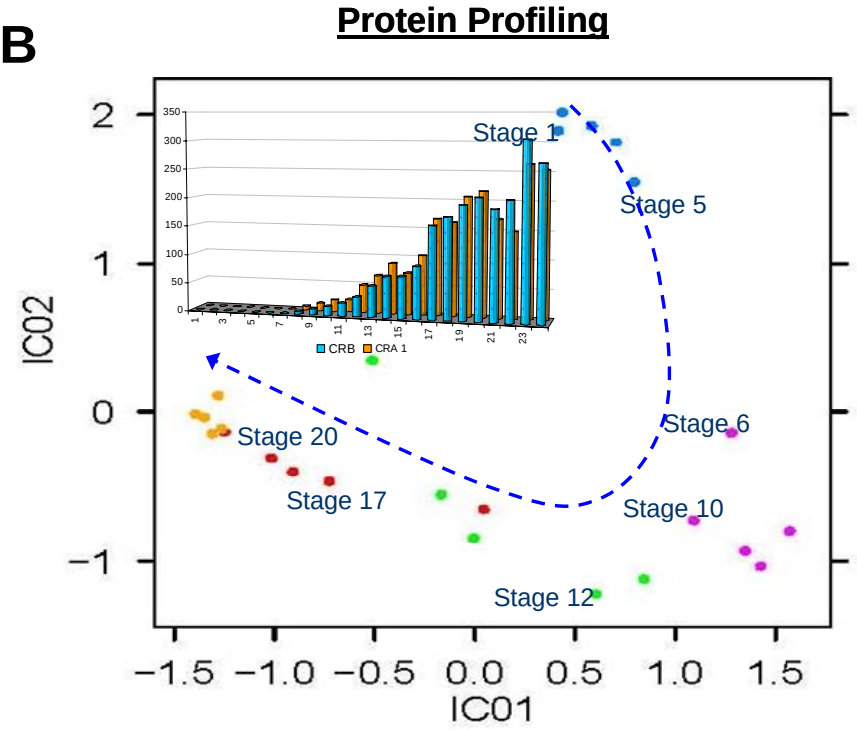
Some of this distant control is likely to be provided by key regulatory genes that control the major shifts in seed composition through development. Figure 19 shows the overall picture obtained in Arabidopsis, summarizing the major

changes in composition that occur during seed development in this model plant. Here major shifts have been noted, with respect to content of metabolites (Figure 19A) and proteins (Figure 19B), as well as in overall gene expression (Figure 19C). The identity of many of the key regulators has now been established in Arabidopsis, as well as in the legume model plant, *Medicago truncatula*. For both of these species, a platform has been set up to allow other researchers to find out what regulators have changed in their plants and seeds. The platforms enable others to ask, for example, which regulators are responsible for plant responses to drought and disease, and to find out which regulators are affected in mutants with altered seed composition.

A



B



C

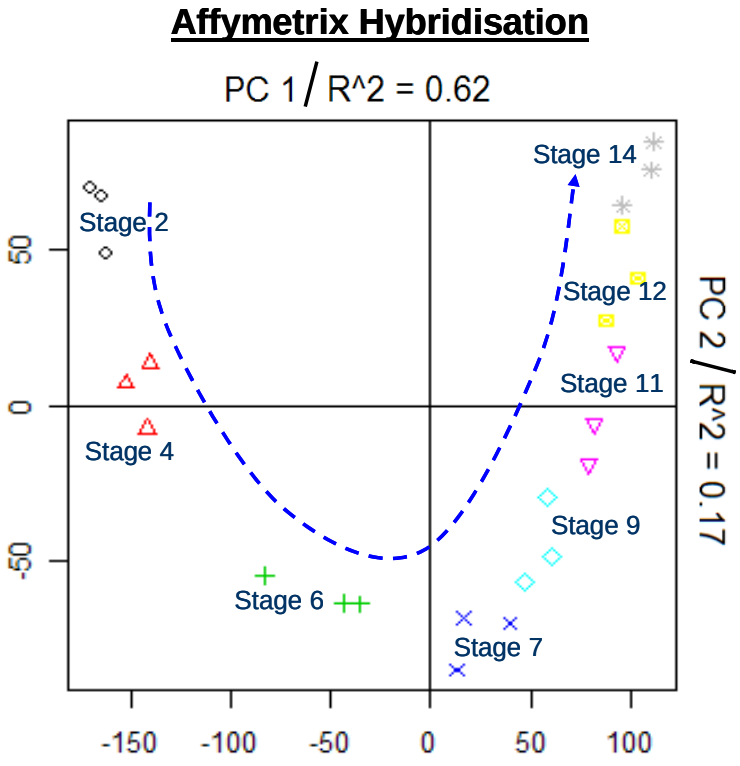


Figure 19. *A summary of the changes that take place during seed development in the model plant, Arabidopsis. Changes in small compounds (metabolites) (A) , seed proteins (B), and overall gene expression (C) are analysed by statistical methods to show groupings that are diagnostic of stages of seed development. Stage 1 represents the smallest and stage 24 the most mature stage analysed.*

14	16	18	20	22	Primer pair	Annotation	BLAST
					C1	CREB	M. truncatula
					C22	GRAS TF	M. truncatula
					S9f	STYLOSA WD40-like	M. truncatula
					C15	Tim10/DDP family zinc finger	M. truncatula
					C90	ABI 3	P. sativum
					C11	Zinc finger protein SHI	A.thaliana
					C86	TFIIH	A.thaliana
					D54	G-box element binding protein	P. sativum
					S9r	STYLOSA WD40-like	M. truncatula
					S13	CCAAT-box TF	A.thaliana
					D23	RPA 70	P. sativum
					D34	AT-rich element binding factor 1	P. sativum
					S12	Leucine zipper	M. truncatula
					C24	Nuclear antigen homolog	Vicia faba
					S1	WRKY DNA -binding domain	M. truncatula
					D16	ABI 3	P. sativum
					D25	heat shock TF (HSFA)	P. sativum
					S18	bZIP TF 6	M. truncatula
					S2	bHLH TF	M. truncatula
					C30	Pentatricopeptide repeat protein	M. truncatula
					H1	MYB112	Glycine max
					C112	AUX/IAA family	P. sativum
					C116	EREBP-like protein	Glycine max
					C98	MYB136	Glycine max
					D39	plastid envelope DNA binding protein	P. sativum
					S7	"NF-Y, CCAAT-binding"	A.thaliana
					H2	bZIP89	Glycine max
					D32	DNA binding zinc finger protein	P. sativum
					C23	B3	M. truncatula
					C53	HLH DNA-binding	M. truncatula
					C56	TFIIB	Glycine max
					C94	PHD finger family protein	A.thaliana
					C18	GAGA-binding protein	Glycine max
					D44	Dof protein ERDP	P. sativum
					D14	DNA-binding protein PD2	P. sativum
					D21	RPA 32	P. sativum
					C109	MYB TF	A.thaliana
					C3	AP2/EREBP TF ERF-2	Gossypium hirsutum
					C37	CBF/NF-Y	M. truncatula
					S14	bZIP128	M. truncatula
					D33	HMG1	P. sativum
					S17	Ethylene-responsive	M. truncatula
					D42	Myb2	P. sativum
					C48	NAM-like protein 6	Petunia hybrida
					C104	NAM-like protein	Prunus persica
					C3a	AP2/EREBP TF ERF-2	Gossypium hirsutum
					C6	HMG 1 protein	P. sativum
					D7	HMG-I/Y	P. sativum
					S10	AT-hook DNA-binding protein PD1	A.thaliana
					D20	SARBP-2	P. sativum
					C5	"Zinc finger, Tim10/DDP-type "	M. truncatula
					S15	MYB62	Glycine max
					C72	TUBBY family protein	A.thaliana
					C95	MybSt1	M. truncatula
					C91	HD-zip protein	Solanum demissum
					S4	RING finger family protein	A.thaliana
					C31	E2F/dimerisation partner (TDP)	M. truncatula
					C50	aux/IAA protein	Populus tremula
					C39	C2H2 zinc-finger	A.thaliana
					C10	B TF 3	A.thaliana
					S3	KNAT4 homeobox protein	A.thaliana
					C20	TFIID	Flaveria trinervia
					C41	TATA-box-binding protein	Glycine max
					C83	KIWI; DNA binding	A.thaliana
					C7	HMG-I/Y	P. sativum
					C16	TFIIE	Oryza sativa
					S5	MYB130	Glycine max
					C110	AN3 (ANGUSITFOLIA3)	A.thaliana
					S6	NDX1 homeobox protein	Glycine max
					D31	Dof7	P. sativum
					D53	MYB-related	P. sativum
					C9	LEC1-like protein	P. coccineus
					S11	"AGL15, MADS box"	Glycine max
					C9a	LEC1-like protein	P. coccineus
					D36	AT-rich element binding factor 3	P. sativum
					D19	SARBP-1	P. sativum
					H8	Actin	P. sativum
					D35	AT-rich element binding factor 2	P. sativum
					S16	B3	M. truncatula
					D46	SOC1a	P. sativum
					C89	TF DP1	Populus tremula
					D47	VERNALISATION 1 b	P. sativum
					H3	Ubiquitin conjugating enzyme	P. sativum
					C40	Scarecrow-like 3	A.thaliana

Experiments are underway in Arabidopsis to alter the amounts of some of the key regulators and to assess how seed composition changes as a result. It is expected that the picture shown in Figure 20 will be altered drastically when the amounts of some of these key regulators are changed.

The challenge then is to identify those regulators with the same function in the model legume, *Medicago truncatula*, and later in legume crop species. Then the equivalent genes may be modified in the crops, using knowledge acquired from the processes described in the models.

As a first step in the model to crop transition, arrays of regulator genes are being prepared for pea. Figure 20 lists some of these and their identity, based on sequence similarity with other species. Pea mutants affected in seed composition are being examined to establish which, if any, regulators may be altered in amount. Figure 20 shows a pictorial representation of the changes (green or red) that have occurred in one such mutant. Here protein and carbohydrate accumulation have been affected, that could, for example, be a consequence of higher amounts of the LEC1-like regulator. All of these studies highlight critical regulators that, once their precise function has been verified, could then be the basis of a breeding strategy for improved crops.

Figure 20**.** *A pictorial representation ('heat-map') of the TFs with altered expression in mutant (transgenic) pea lines, compared with wild type. TFs with lower expression are shown in green, whereas those up-regulated are shown in red, for 5 stages of seed development.*

Module 4: To identify the factors affecting grain legume seed composition and supply

This module aims to understand factors that impact on the growth and productivity of grain legumes. The module takes a variety of approaches and includes a substantial effort on the understanding of the relationship between corresponding biological processes in crops and models by exploiting technical developments in the model system *Medicago truncatula*.

As for many crops the constraints on grain legume productivity come from several sources: environmental stresses such as response to water deficit (addressed in WP4.1), pests and diseases (addressed in WP4.2), and plant and canopy architecture in relation to the timing of seed set (addressed in WP4.3). The understanding of how crops, as stands of plants, acquire, use and partition nutrients to seeds is of fundamental importance for agriculture and within WP4.4 we have made a concerted effort to generate plant physiological models that will identify parameters of critical importance in the N economy of crops.

Module 4 has close ties therefore with Module 5 that provides genetic and genomic tools for these analyses; with Module 3 in relation to the partitioning of nutrient to seed and with Module 2 in relation to the performance of crop stands.

In general our aim has been to find and understand how best to use available genetic variation. Where this does not seem to be available in our crop species we have sought to understand the underlying genetic mechanisms in a closely related model species so that in the future we may be able to generate new genetic variation or uncover hidden sources of useful variation.

The module analyses mutants, variants and transgenic plants with a range of tools, often whole genome genetic or transcriptomic approaches as well as open searches involving proteomic and metabolomic studies. The Module has coordinated activity in model and crop species in order to make lasting links between researchers and between systems that have reciprocal strengths.

Figure 21
Pea field in Essex, UK

WP4.1 Abiotic Stress

Knowing the pattern of activation of gene expression during environmental stress will lead to a better understanding of the interrelationships of the multiple signalling systems that control stress-adaptive responses in legumes. Transcriptome studies in *Arabidopsis thaliana* assessed gene expression changes after cold, drought and salinity treatments. This work revealed that 11% of the stress-inducible genes are potential transcription factors further confirming the relevance of gene regulation in stress adaptation.
In this work package we aimed to dissect the mechanisms dealing with abiotic stress tolerance in agriculturally important grain legumes, by analysing the responses induced by diverse environmental stresses in pea, chickpea and *M. truncatula*. As significant progress has been made at the genetic and genomic levels using the model legume *Medicago truncatula,* we had access during the project to the different functional genomic tools developed for this species and we contributed to the development of a new platform of 1000 transcription factors from this model legume.

The objectives of WP4.1 were:
To characterise the transcriptomic and metabolomic changes occurring under environmental stresses in chickpea, pea and *Medicago truncatula.*
To identify critical regulators of gene expression and metabolic adaptation using functional genomic approaches and reverse genetics in the model legume.
To assess the effect of QTLs on gene expression patterns in *Medicago truncatula* and chickpea using ecotypes with contrasting responses to environmental stresses.
To produce a cDNA microarray with diagnostic legume stress responsive genes

During the project, to reach our first objective, we characterised the transcriptomes of *Medicago truncatula*, pea and chickpea and defined the expression patterns of a large number of transcripts during the response to environmental stresses. This was done using microarrays and SSH (substractive suppression hybridisation) cDNA libraries and more recently SUPERSAGE, a global approach developed by, Gene-X-Pro (Partner 68). This was coupled with detailed analysis of the changes in the metabolome in *M. truncatula* and pea using innovative technologies such as Nuclear Magnetic Resonance (NMR). These analyses allowed exploring gene regulatory networks and changes in metabolic fluxes occurring in pea, chickpea and *Medicago truncatula*. Indeed, we have identified several hundred candidate genes that were targeted by environmental stresses. For certain cases, notably in *M. truncatula*, we could identify transcription factors regulating those genes through different functional approaches (overexpression and RNAi) such as the Krüpel-like transcription factors. Finally, the SUPERSAGE approach enormously boosted the sensitivity of the transcriptome analysis and a database was constructed to identify those genes that were exclusively expressed under stress conditions. These genes could constitute excellent markers to monitor environmental stresses *in planta* under field conditions and was the basis for the development of a Legu-CHIP by Gene-X-Pro in the project (see below). In relation to metabolome changes occurring during abiotic

stresses, large scale NMR analysis from a variety of samples from pea plants submitted to drought stress revealed unequivocal and consistent metabolite markers of drought stress such as trigonelline, valine, proline, homoserine, gamma-aminobutyric acid (GABA), isoleucine, leucine, and myo-inositol. This was coupled with detailed analysis of carbon fluxes in response to water stress in grain legumes and *M. truncatula* ecotypes, revealing interesting metabolic differences linked to their differential response to environmental stresses.

In parallel to these global analyses, we characterised several ecotypes from *M. truncatula* and chickpea showing contrasting differential responses to environmental stresses (tolerance and sensitivities to salt and drought stress). A QTL (quantitative trait loci) approach was developed to define genetic loci responsible for this behaviour in response to environmental stresses. Comparing transcriptome responses in an integrative way in model and grain legume species coupled to QTL characterisation at the genetic level allowed us to define control mechanisms exerted by the QTLs on gene expression patterns. This also revealed specific candidates to be key regulators of these environmental stress responses by combing physical and genetic maps in order to co-localise QTLs to specific genes. Although the work in chickpea was delayed due to several problems in the growing seasons, in *M. truncatula*, a specific TF was found to co-localise with a QTL controlling root growth under salt stress and functional studies are now being carried out to further validate the role of this regulatory gene in salt stress tolerance.

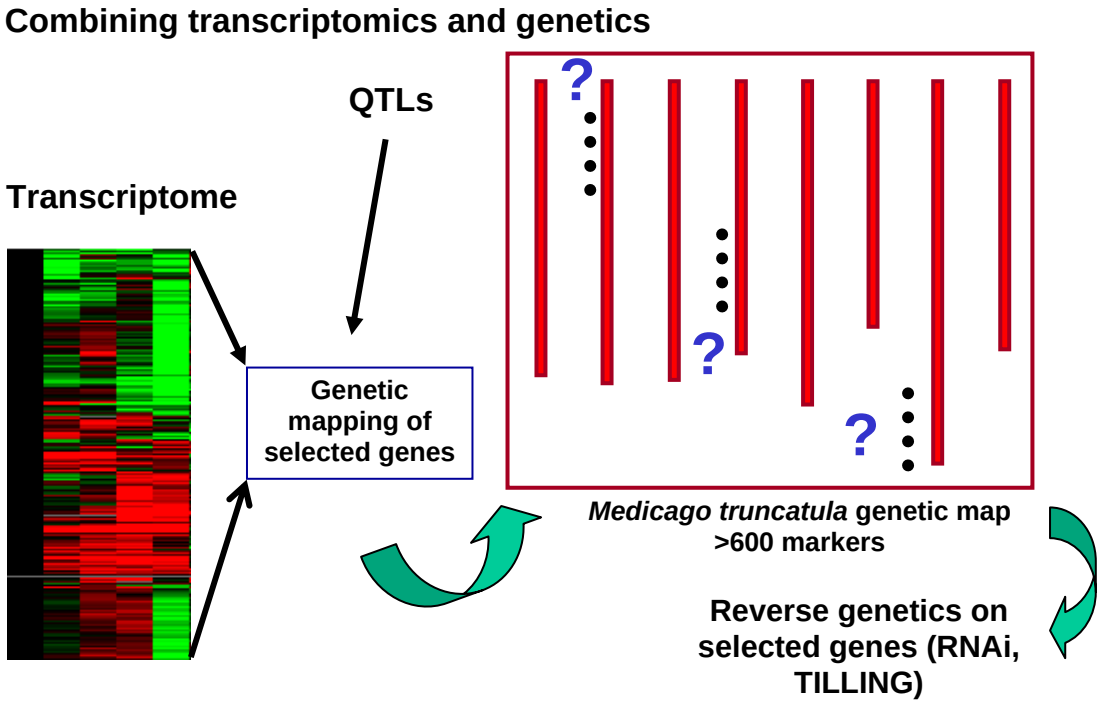


Figure 22. Coupling QTLs and transcriptomics lead to the definition fo candidate genes for reverse genetics studies to identify their role as critical regulators of environmental stress tolerance in grain legumes.

These studies were completed by parallel developments in Module 5 on different platforms for functional genomic analysis, most notably the TILLING platform. WP4.1 profited from these developments to identify several alleles of different genes studied in the context of environmental stresses. Indeed, an RNAi approach initially revealed a role of a new Receptor-like Kinase (identified in the transcriptome analyses) in the determination of the root susceptibility to salt stress. By identifying two TILLING alleles for this gene in *M. truncatula*, we could demonstrate a critical role of this receptor-like kinase in the activation of salt-dependent signalling pathways and in the perception of the soil environment. Other candidate genes have also been TILLED and will be further analysed in the future. This highlights the impact of global genomic approaches for the identification of key genes dealing with environmental stress tolerance.
By the end of the project, through careful bioinformatic analysis of the database made by GeneXPro, a workable version of the LeguSTRESS-DNA chip was launched. The LeguSTRESS-chip was used for hybridisation data on different recombinant inbred lines derived from the QTL analysis and was evaluated for its ability to differentiate between stress-tolerant and susceptible plants. This turned out to be more difficult than expected but development of this chip will continue at GeneXpro after the end of the project. In addition to this application, the Legu-stress DNA chip will be assayed for their capacity to monitor the degree of stress that a legume plant is suffering before appearance of stress-related symptoms. This could be directly used in the breeding industry to speed the selection process using these markers and eventually in the field to modify irrigation conditions in a cultivated legume.

A global description of the project is shown in Fig. 23.

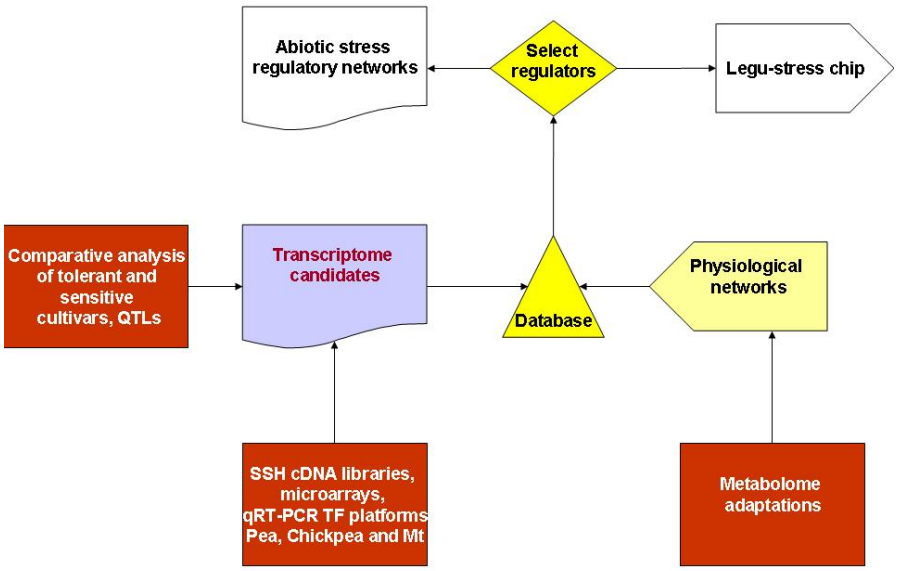


Figure 23: Pert Chart of WP4.1 to identify selected regulators of environmental stress tolerance in grain legumes for development of the Legu-Stress chip and define the regulatory networks involved.

WP4.2 Biotic Stress

We anticipate that a better understanding of plant responses to biotic stresses will allow the development of tools to tackle disease, ultimately ensuring stable yields for farmers cultivating legume crops. This work package undertook both genetic and gene and protein expression profiling studies to identify key regulators of resistance to these diseases. The model legume *Medicago truncatula* was used to gain insights into both molecular and genetic resistance components against major grain legume diseases. A novel technology named superSAGE analysis was developed by using chickpea infected with *Ascochyta rabiei* as a new way of generating high throughput genetic and genomic data for a relatively poorly studied crop, complementing results obtained from model systems. All the plant / pathogen studies developed throughout GLIP will help to provide new target genes that will allow the improving of disease resistance and control in crop legumes.

Throughout GLIP, studies were notably focused on three major pea pathogens: *Aphanomyces euteiches*, responsible for root rot on several legumes and which causes the most severe economic losses on pea in France and Northern European countries, *Mycospharella pinodes*, responsible for Ascochyta blight on pea, of worldwide distribution, and *Orobanche crenata*, a parasitic plant responsible for severe infection in Southern and Mediterranean countries. Besides being of huge economic importance, these three diseases are characterized by having a complex inheritance and for being difficult to control.

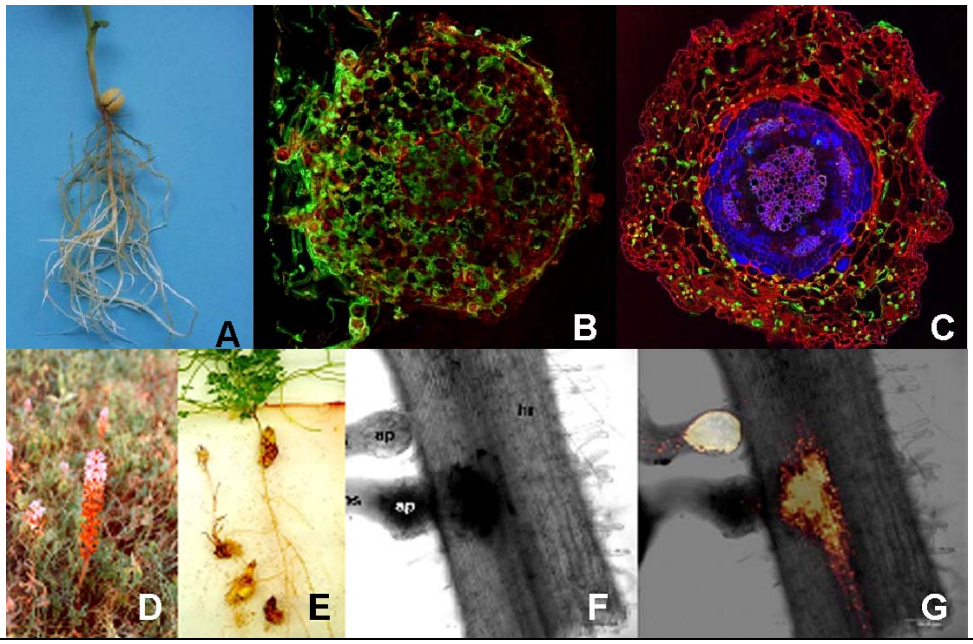


Figure 24: Interactions between *Aphanomyces*, broomrape and their host legumes.
A-C: A) Aphanomyces root rot symptoms on pea. B & C): Observations using confocal laser microscopy of transversal thin root section of susceptible (B) and resistant (R) *M. truncatula* lines. (green: *A. euteiches* mycelium, red: cell wall; blue: phenolic compounds)
D-G: *Orobanche crenata*: D) *O. crenata* on pea; E) Unusual *O. crenata* infection on *M. truncatula*; F) Incompatible interaction of *O. crenata* on Mt196 accession of *M. truncatula* (transmission). A darkening of the attachment organ and root tissues can be observed. G) showing intense fluorescence in the host central cylinder and in the youngest attachment organ.

A search for resistance genes that might be introduced through conventional breeding programs was undertaken. To accelerate this quest, model pathosystems were established with these parasites in interactions with *M. truncatula* genotypes. The wealth of genetic and genomic resources available within this species allowed us to identify new sources of resistance as well as molecular and genetic components involved in resistance mechanisms (examples of such mechanisms are illustrated in figure 24).

Final GLIP results of WP4.2 indicated that many initial objectives have been reached. New resistance sources have been discovered, some resistance mechanisms have been elucidated and on the pathogen side novel targets for pesticides were identified. Many of these results can now be transferred to cultivated legumes and should therefore provide new solutions for breeders and legume farmers to better protect their crops.

WP4.3 Plant Architecture

Within this module we aimed to characterise and isolate genes involved in the regulation of plant architecture, mainly in pea, and to characterise some of the physical parameters that contribute to lodging in this crop. To do this we have combined a range of methods including comparative genetic mapping, direct screening of mutant populations, candidate gene approaches as well as accessing the GLIP TILLING platforms, the use of transgenic plants and the virus induced gene silencing system developed for pea by Ole Lund's group in Denmark (outside the GLIP consortium).

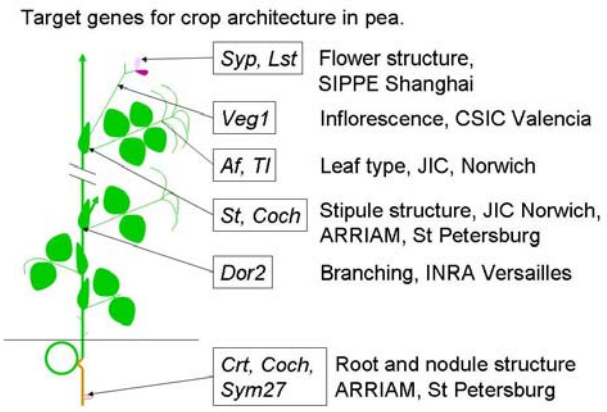


Figure 25 Target pea genes

Two well known genes that regulate the development of the pea compound leaf *afila* (*af*) and *tendril-less* (*tl*) (see below) have been targets for cloning and for both the corresponding structural genes have been identified with confirmation of gene function through the pea TILLING service at URGV in Evry completed for *tl* and currently underway for *af*.

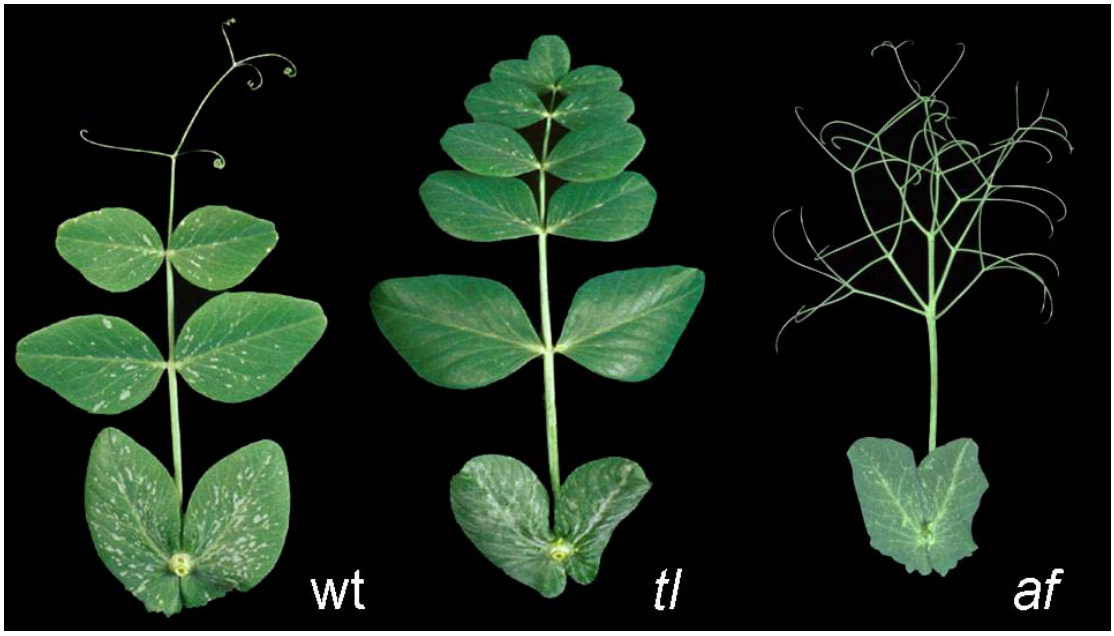


Figure 26The form of pea leaves on wild type (wt) plants as well as *tendril-less* (*tl*) and *afila* (*af*) mutants is shown.

The control of flower formation is an important process in crop productivity, and before the advent of a major effort in arabidopsis research, pea was a key

genetic and physiological model system for the dissection of this process. Accordingly many mutants of pea have been identified that affect this process. One unique mutant *vegetative1* (*veg1*) fails to flower under any circumstances and represents a failure to form that component of the inflorescence from which the flowers are derived, but the mutant phenotype can be rescued in part by another mutant that effects another homeotic transformation within the inflorescence.

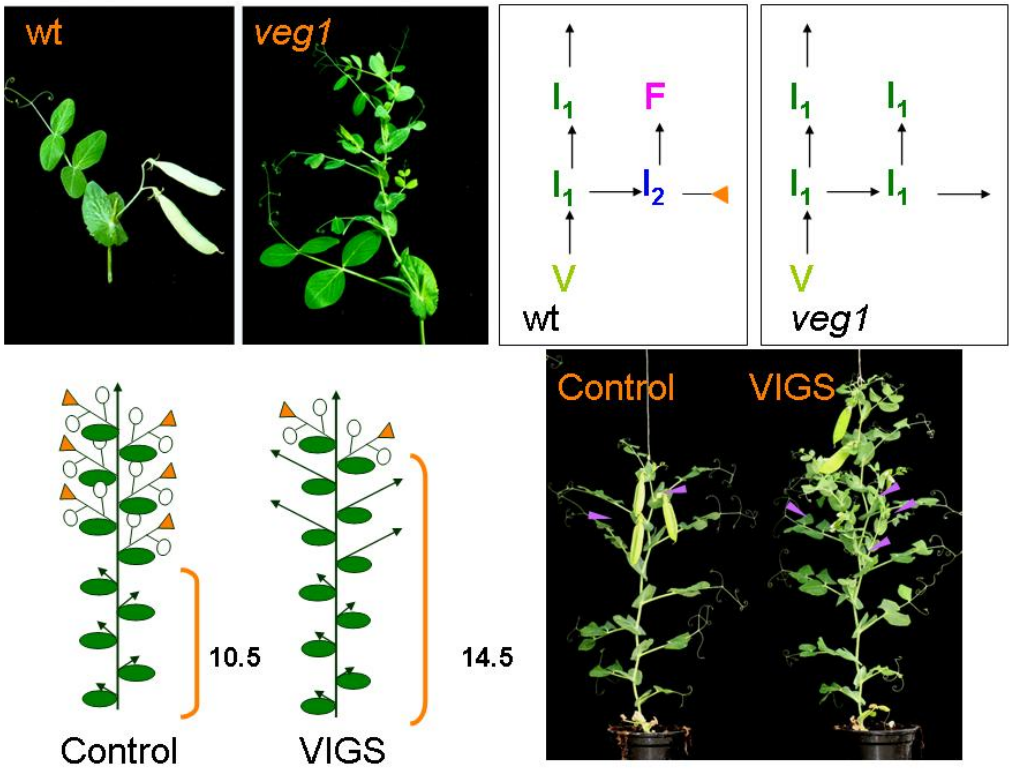


Figure 27 The *veg1* mutant fails to generate secondary inflorescence meristems (I_2) from the primary inflorescence (I_1). As flowers are derived from I_2 the *veg1*mutant fails to produce flowers. In a virus induced gene silencing experiment (VIGS) suppression of wild type *Veg1* expression results in a delay in the onset of flowering by about four nodes.

Lateral vegetative ('V' above) bud outgrowth is regulated by a set of genes designated *ramosus1* to *ramosus6*, but the failure of bud outgrowth in these mutants is dependent on other gene functions. In the *ramosus4* mutant normally quiescent buds grow out prolifically giving a highly branched stem, the *dormant2* mutant restores a low level of cell cycle functions (such as cyclin gene expression) in an *rms4* mutant background and thus restores the dormant wild type phenotype.

WP4.4 Carbon/Nitrogen Allocation and Seed Quality

At the end of the growth cycle, symbiotic N fixation decreases and is highly sensitive to environmental factors. Although mineral N root absorption could then complement symbiotic N fixation for N acquisition of the crop, the shallow root system of grain legumes constitutes a limitation to nutrient uptake. Thus, N absorption declines during seed filling and cannot sustain the high seed demand, which provokes N remobilization from vegetative tissues. This process interacts with photosynthesis and induces senescence, which reduces the seed filling period. Hence, improving C and N flux towards seeds during seed filling in order to optimize the yield level and its quality relies for a large part on either reduced dependency on N remobilization or enhanced N uptake efficiency.
The objectives of the WP were to i) to acquire knowledge concerning fundamental mechanisms involved in N and C retrieval by the plant and their redistribution towards harvested organs, ii) to integrate the knowledge in plant ecophysiological and genetic models by quantifying the effects of the environment and of the genetic background on these elementary mechanisms, iii) to identify the molecular basis involved in key traits in order to provide guidance for building ideotypes and develop tools for marker assisted selection. In this workpackage, an integrative biological approach, relying on the co-operation between agronomists, ecophysiologisst, plant physiologists, molecular biologists and geneticists was undertaken.

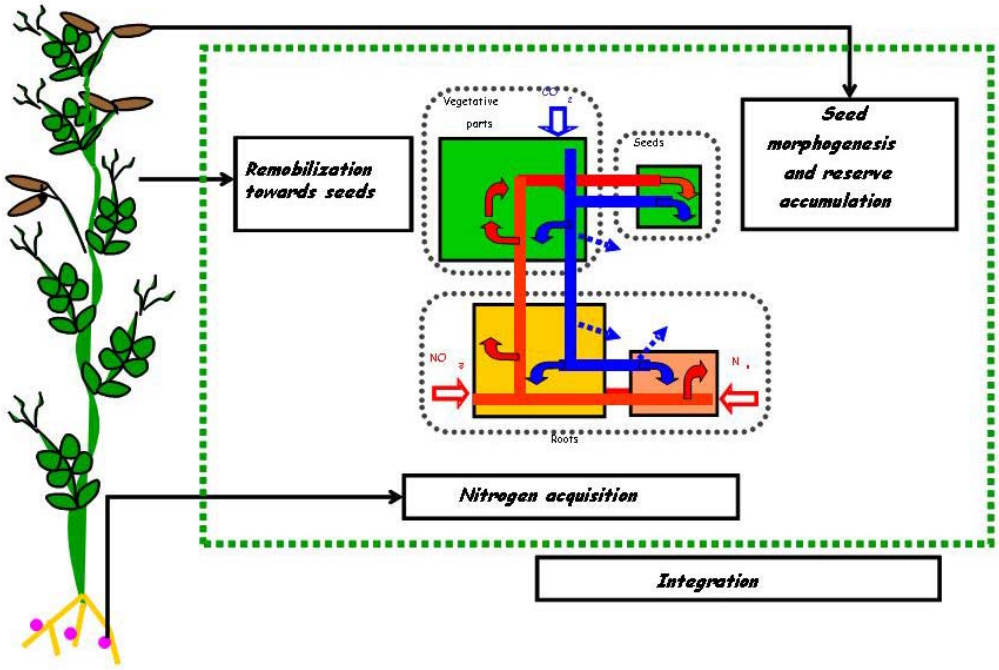


Figure 28: Main research axes in WP4.4. Knowledge of these physiological mechanisms was then integrated with ecophysiological to evaluate the relative importance and to describe the relations between the plant in field conditions and its physical environment for different genetic constructions.

Three main analytical research axes have been defined for understanding the key mechanisms involved in plant performance and its modulation by the environment.

1) The determinant effect of **nitrogen nutrition** on yield elaboration and its quality makes it a key step for crop production. Transcriptomics analyses were performed for a ***global view of gene networks for nodule formation and activity in M.trunculata***. Results confirmed the reliability of microarray and large scale qRT-PCR results obtained during GLIP. A robust view of the symbiotic transcriptome has been generated, with genes (about 2000) found to be differentially expressed during nodulation, including putative transcription factor genes (approx. 160), which represents a major achievement toward the analysis of symbiotic regulation networks. Important ***regulators of the nodulation process*** were then identified and characterized, providing clues on their biological importance and possible functions. Three genes (*MtMMPL1, MtHAP2.1, MtEFD*) have been characterized in more detail by a combination of molecular and reverse genetic studies. Transcriptomics has proved to be fully complementary to forward genetics to identify important players and regulators of nodulation. The work carried out during the GLIP project has notably provided the first examples of transcription factors important respectively for nodule meristem and for bacteroid and plant cell differentiation. Interesting perspectives are opened up to understand the mechanism of action of these transcription factors, in connection in particular with protein complexes and phytohormone regulation.

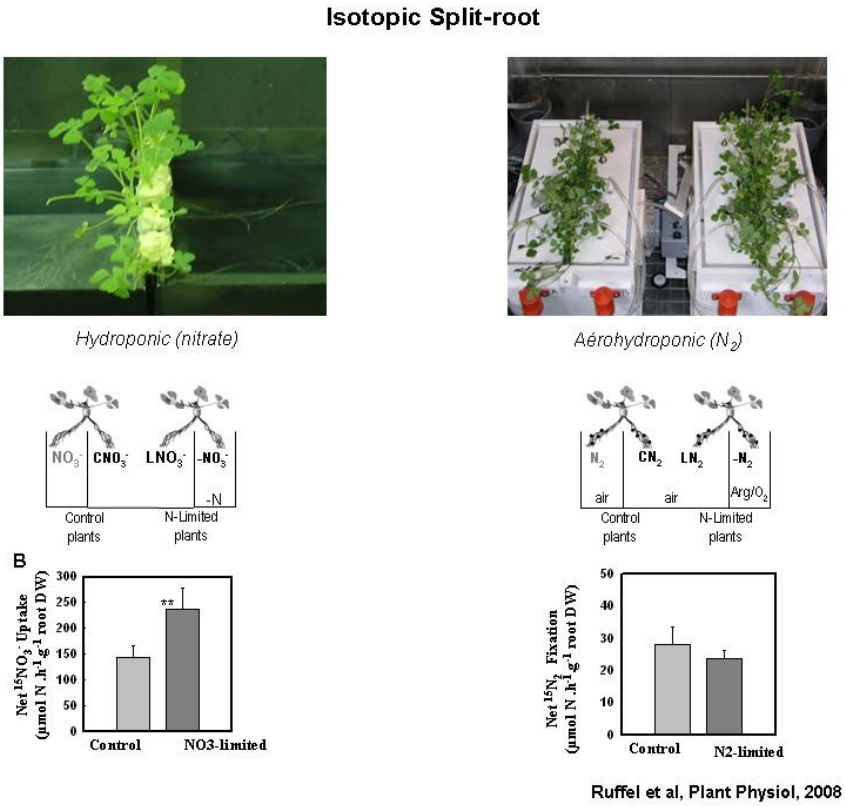


Figure 29. Comparison of Control and N-limited plants. Split-root systems were used to compare N-limited and control plants. NO_3 (1 mM) or N_2 fixing plants compared after 4 days treatment. Controls the same regime on both sides of the root system (C-roots) or with the N source removed to the treated side.

The ***adaptative response of the nitrogen nutrition pathways*** (NO_3^-, $NH_4^+$and N_2) to a variation in N availability has been studied using split root systems and a combined approach driven by ecophysiologist and plant physiologists. The three acquisition pathways of *M.truncatula* have been found to be controlled by feedback repression which may prevent the plant to acquire N in excess. This repression is likely to be the result of a feedback repression exerted by downstream N metabolites on N acquisition pathways as it has been suggested in *A. thaliana* for NO_3^- uptake. This result suggests that the regulatory mechanism that senses the N demand may be common for the three acquisition pathways. However, these three acquisition pathways do not display the same ability to compensate rapidly to a local N limitation. Only NO_3^- acquisition may allow the whole plant to maintain its growth under such conditions. Molecular responses associated with these physiological responses have been characterized by a transcriptomic approach. A long term response to N limitation has been found in the case of N fixation involving the development of new nodule structures that may compensate partially for the deficit after a delay. Whether the mechanisms controlling the development of new structures for N acquisition are connected or not to the mechanisms controlling the activity of these structures remains to be determined.

An ***integrative approach,*** using whole plant C/N flux measurements, transcriptomics and proteomics, ***probed the regulation of nodule metabolism***. Genetically modified *M truncatula* plants deregulated for the synthesis of root nodule glutamine synthetase GS were used as a tool to evaluate the regulatory role of (GS) in terms of N use efficiency and yield. An expression profile revealed several interesting genes potentially implied in the regulation of nodule nitrogen metabolism were identified as differentially expressed in both plants over expressing GS and in antisense plants. Transcriptome analysis identified many interesting genes including genes involved in nitrogen and carbon metabolism, in membrane trafficking and transport, in post-translational protein regulation, signal transduction and transcriptional regulation, that would not be identified otherwise. Metabolomic studies in root nodules of *M. truncatul*a highlighted a small number of metabolites that could be related to metabolic enzymes involved in their biosynthetic pathways also found to be significantly altered by the transcriptome analysis. Glutamine synthetase is an important component of Nitrogen Use Efficiency and plant yield. The *M. truncatula* plants over-expressing GS specifically in root nodules display an enhanced growth phenotype as quantified by increases in biomass and seed production, increased nitrogen fixation activity and increased nitrogen utilization efficiency. Thus, it may be possible to increase plant productivity by the manipulation of specific GS genes. From ***previous ecophysiological studies, an ideotype*** (having late establishment of nodule morphogenesis, with an early and rapid initial root growth) was defined for legume breeders in order to optimize plant N nitrogen acquisition. In ***order to build this ideotype***, candidate genes of root development were searched through a *Tnt1* mutagenesis program performed on *Medicago truncatula* (WP5.2). Putative root phenotype mutants were screened on root, nodules, shoots. Contrasted mutants were selected: one “high root branching” mutants having lower nodulation, reduced shoot growth and three “long root” mutants with higher root and shoot biomass, standard nodulation. Potential tagged genes should be identified in mutants of interest.

2) Nitrogen and carbon remobilization from vegetative parts towards seeds constitute an important part of total N invested in seeds. Both the level and the rate of N remobilisation are determinant for i) nitrogen reserves accumulated within seeds and ii) maintaining a photosynthetic activity during the late growth cycle in order to sustain the C needs of the plant. The contribution of N remobilization to seed N yield during the seed-filling period was determined in various pea genotypes using ^{15}N labeling. Our results displayed a genetic variability for source organs for remobilized N and for the efficiency of N remobilization. ***Proteomic, metabolomic and flux measurement*** gave an ***integrated view*** of the changes in metabolic pathways in vegetative and reproductive leaves during seed filling. A proteome reference map of mature leaves from the broad-leafed genotype Caméor was established and compared with proteome data obtained from vegetative and reproductive leaves during the seed filling period to identify the activated or repressed metabolic pathways involved in the N remobilisation process. Different proteins implicated in a variety of cellular functions, most of them involved in energy/carbon metabolism were identified. Quantitative proteomic analyses of leaves of vegetative and reproductive nodes carried out throughout the seed-filling period highlighted proteins that were remobilised and involved in N remobilisation. These proteomic analyses were completed by a dynamic measurement of total N, protein, amino acid, nitrate, total C, starch, hexose and sucrose contents in several strata of vegetative organs at different dates of seed development. The older leaves reaching a late stage of senescence during the seed filling period have a metabolism mainly pointed towards structure dismantlement and macromolecule degradation into exportable elements such as glutamine and sucrose to seeds. By contrast, in younger leaves, starch remobilization can supply the exported flux of sucrose and amino acids coming from protein degradation that would be exported to seeds rather than being used as carbon skeletons for sugar synthesis. Proteins were the major source for N remobilization, but amino acids and nitrates could constitute additional N supplies for filling seeds and would play a role in the regulation of the process of N remobilization. The use of biochemical measurements and quantitative proteomic analyses complmented by quantification of N fluxes throughout the seed-filling period gave a first, global and dynamic view of the potential metabolic pathways that are activated during N remobilization in pea plants.

3) **Seed morphogenesis and reserve accumulations** depend not only on assimilate supply but also on the genetic background and the growth potential limited by the physic and trophic environment. Seed growth potential is highly correlated to the number of cotyledonary cells, established early during seed morphogenesis. An ***analysis of seed potential*** modulation by the environment, according to the C and N flux reaching seeds during the early phase of cell division, has been driven using i) the use of labeled carbon to measure C fluxes within plants, ii) varying CO_2 enrichment of the air to vary the C fluxes through photosynthesis and assess the effect on C reaching seeds; iii) simultaneous measurement of the amplitude and duration of seed mitotic activity using flow cytometry. A Michaelis Menten like kinetic relation was found between the flow of sucrose reaching seeds during the cell division period and the activity of enzymes involved in the cell cycle (cyclins). Studying the ***influence of seed sink strength*** on the extent of N remobilizations and

exogenous N supply is undertaken via functional analysis of transgenic material whose metabolism and seed sink strength have been modified. Study of the over-expression of an amino acid permease from V. faba, VfAAP1, in *Vicia narbonensis* seeds demonstrated that seed sink strength for nitrogen, seed protein concentration and seed weight were increased by the higher capacity for amino acid uptake. Increased seed sink strength for N stimulates seed and vegetative growth which ultimately leads to a higher ratio of vegetative to seed biomass at maturity and thus a lower harvest index.

In order to define key bottlenecks (in C and acquisition, N remobilization and seed potential) for high seed quality and sustainable agriculture, knowledge on physiological mechanisms and the various identified genetic components was integrated in ecophysiological models i) of the plant development calendar, useful to predict which organs are competing for C and N assimilates at each time of the growth cycle, ii) of C and N acquisition, allowing to establish C and N fluxes towards the competing organs. iii) to describe the relations between the plant in field conditions and its physical environment for different genetic constructions

An ***ecophysiological model*** allowing the simulation of the ***relations between leaf area and N retrieval*** by roots has been developed in order to allow the prediction of the impact of environmental conditions on plant growth and so would constitute a decision tool in order to choose adequately the environmental conditions before sowing plants. The model also allows estimating *a posteriori* variables that were not measured in real time during the time course of the growth cycle. The model will allow the systematic characterization of phenotypic variability i) originating from the environment by determining how intermediate variables are affected by the environmental factors, ii) related to genotype. The model could constitute an innovative tool for diagnosing the differential N retrieval between lines. A ***methodology was developed to assess the agronomic and environmental interests of new winter pea cultivars*** before marketing, and to determine the characteristic that should be combined to guarantee these advantages. A dynamic pea crop model has been elaborated and predicts many aspects of plant (including shoots and roots) development, plant growth, yield component and plant N and water consumption at the plot scale. This crop model gives a good account of the effects of sowing date, genotype (particularly winter, highly reactive to photoperiod or not, and spring varieties) and soil structure. The model was used to assess different potential candidate cultivars by being coupled with one model of work organisation at farm scale and with a model simulating soil structure evolution according to the soil water content. This work increased the knowledge on root system development for different sowing and soil structure conditions. A crop model for spring and winter cultivars and sowing period, which takes into account the variability of nitrogen availability and soil structure, is now usable. Finally, the combined uses of different models showed that the results of the assessments of potential cultivars were different depending on whether constraints at the farm scale were taken into account or not. The whole work proposes to breeders potential cultivars that present agronomic and environmental interests in farm.

The ecophysiological model describing the relationship between the environment and plant behaviour allowed defining input variables and parameters for multi-trait QTL studies.

The relevant parameters to measure root and shoot development, N accumulation and partitioning in the plant were determined, evaluated in a field trial on the most suitable *M. truncatula* RILs populations. An ***innovative approach*** was initiated which consists in establishing the ***synergy between genetics and ecophysiology***. The QTL research has been applied, on phenotyping data acquired on a population cultivated under two nitrogen nutrition regimes and on genotyping data, on two types of traits : (1) QTL for integrative traits (e.g. biomass, amount of acquired N); (2) QTL for "efficiency traits" arising from ecophysiological analysis (biological efficiency, specific N retrieval and efficiency of conversion of N in foliar surface). *When symbiotic fixation is the main source* of N for the plant two major QTL have been located and colocalized with candidate genes : a gene involved in symbiosis (MtSym6); a gene involved with N_2 symbiotic fixation (MtLb1). This confirmed the hypothesis that RILs had contrasted abilities to fix symbiotically N_2, with major consequences on plant functioning. *When nitrate assimilation represents the main N supply* for the plant three chormosomal regions seem to be implicated in phenotyping differences between RIL. The positioning of those QTL related to efficiency variables allowed to define hypothesis regarding the underlying genes: a gene involved in nitrate assimilation which has consequences on biomass accumulation ; a gene involved in carbon assimilation which affect biomass and nitrogen accumulation.

For pea**,** several clusters QTLs with co-location between root or nodule development and nitrogen nutrition were found. Therefore, this Quantitative Trait Loci approach allowed a better understanding of the relationships between them. Moreover, the construction of a pea genotype with increased root system but not depressed N-fixing activity should be possible. Work will continue by confirming the impact of the root or nodule QTLs in Nitrogen Nutrition in other populations or environments. Later on, the synteny with *Medicago truncatula* will be used to identify candidate genes of root and nodule development.

MAIN CONCLUSIONS:

Tools now available in *M. truncatula* for functional genomics and reverse genetics (RNAi, EMS tilling, deletion and insertion mutants) make the functional analysis of candidate genes much more efficient and powerful. This is essential to fully understand the formation and function of the organs relevant to agronomical and ecological properties of legumes. Understanding and modelling the genotypes/environnement interactions of mechanisms involved in crop production and quality is a major goal for us to be able to exploitat available genetic variability and/or guide gene introgression. A better match of the genotypes to different environments will allow the stabilisation and increase of crop production and thus eventually to enlarging the legume crop area.

Ecophysiological models allow the deconvolution of integrated processes into their constituent parts. Our results demonstrate the value of coupling ecophysiological research with quantitative genetics to improve the sensitivity of QTL detection, and also for interpreting the role of underlying genes.

Module 5 Genetic and genomics tools

One of the reasons why legumes are undercultivated in Europe is the weakness and irregularity of their yields due to the fact that legumes have been much less genetically studied and bred than cereals and oil-yielding plants. The objective of Module 5 has been to devise a series of innovating genetic and genomic tools to facilitate studies and breeding of legumes.
Legume crops have large and/or complex genomes making their analysis difficult. This is why *Medicago truncatula*, an annual medic, having a small and simple genome has been chosen as a model. Previous studies in the frame of a FP5 European project had revealed that the gene order on the chromosomes of *Medicago* and pea, the major European grain legume crop, is strongly conserved, in spite of the very large difference in their genome size (500 versus 3,000 Mb). Major European legume crops, pea, broad bean, chickpea, lentil, lucerne and clover are evolutionarily and genetically closely related to *M. truncatula*. The driving idea of this module was thus to exploit this genome conservation between model and crop species to develop a series of diverse and complementary novel tools.

WP 5.1: Sequencing the M. truncatula genome
Determining gene order on the chromosomes of the model species

As a prerequisite for all the other genomic approaches, this WP focuses on sequencing the euchromatic (gene-rich) part of the *M. truncatula* genome. This sequencing project has been achieved as part of the International Medicago Genome Initiative, a consortium of researchers from the EU (partly funded through this integrated project) and the USA (partly funded by NSF). The sequencing strategy which was chosen, relies on the construction of a robust physical map, which ensures a very solid determination of gene order on the chromosomes. The project is expected to be completed by the end of 2008 and has already identified and localised more than 40,000 genes. Identifying and ordering most genes in a legume species, for the first time, provides a considerable source of information which will radically transform genetics and breeding of legume crops.

WP 5.2: Mutagenesis and reverse genetics
Creating resources to elucidate the function of gene products

The aim of this WP was to make possible the elucidation of the function of gene products by generating resources for the systematic search for mutants in genes of interest in *M. truncatula* and pea. Three complementary mutagenesis strategies were developed. All these mutant collections can be used for both forward (identification of the mutation by scoring at the phenotype) and reverse genetics (identification of the mutation by high throughput screening for DNA modification in a candidate gene). Each mutagenesis has specific characteristics.

(1) Systematic generation of knock-out mutants by insertional mutagenesis in *M. truncatula* using the *Tnt1* retrotransposon.

The advantage of this approach is that mutations are tagged by a specific sequence of DNA, the transposon, which greatly facilitates the isolation of the

mutated gene. More than 5000 mutant lines were obtained, expected to carry around 75000 insertions. This mutant collection could serve for the massive sequencing of insertion borders, providing a great tool for *in silico* reverse genetics.

(2) Deletion mutagenesis in *M. truncatula* using fast neutron bombardment.

This type of approach aimed at providing a resource of mutagenised lines for reverse-genetics and the isolation of knock-out mutants in genes of interest. The detection of the important DNA alterations (due to deletions) makes possible the development of high throughput screening for mutations. A collection of 156,000 M2 mutants has been created and used to identify knock-out mutants in important genes.

(3) TILLING in *M. truncatula* and pea

Chemically-induced EMS mutagenesis can provide a variety of mutant alleles with various effects on the gene of interest (more allelic diversity than with the knock-out approaches described above). Mutations in a given gene are screened by a high throughput technique making use of specific enzymes. Mutagenesis has been performed on pea (4,700 mutant lines) and *M. truncatula* (4,000 lines).

An interactive TILLING database was developed, with two main types of data, the morphological phenotypes of the mutants and, when available, sequences corresponding to the altered gene sequence responsible of the mutant phenotype. This database may be searched using either of two entries: a sequence for reverse genetics strategies, or a phenotypic feature for forward genetics screening.

WP 5.3 Gene expression profiling

Developing standardized methods to identify genes potentially involved in the control of agricultural traits

The major objective was to establish an expression profiling platform to provide microarray tools, hybridisation facilities and expertise for the GLIP participants and for the European legume community at large. Microarrays were prepared with oligonucleotides, for 16,000 genes for *M. truncatula,* and 6,000 genes for pea. These arrays have been used to analyse expression profiling in various organs and physiological conditions in both the model system and pea. In close collaboration with partners of Module 6 (Bioinformatics), procedures have been devised to ensure that hybridisation data from all participants can be uploaded through a common system into the central project database EMMA2, allowing efficient data evaluation and data mining. The existence of a central database for gene expression data is unique in the legume community. Work is in progress to develop web services (BioMoby) to allow an easy extraction of expression information also for non-expert users.

Transcription factors play a key role in the control of plant development and functioning. A set of 1084 *M. truncatula* genes encoding transcription factors has been selected to set up a real-time RT-PCR platform, allowing the identification of a number of important regulators involved in the process of seed formation, plant-pathogen interactions and symbiotic nitrogen fixation.

WP 5.4 Crop and comparative genomics
Defining the rules to transfer gene order information from the model to crops.

The main objective of this WP has been to carry out comparative genetic mapping of the model legume *M. truncatula* and the major European grain legumes to facilitate the transfer of information from the model system to crops. The general strategy was to try to map the same genes in the various species. Crop legumes such as pea, faba bean, chickpea, lentil and clover are closely related to *M. truncatula*. Mapping data confirm the conserved macrosynteny existing within species of this clade, which should facilitate the transfer of information on gene order gained in *M. truncatula* to these major European legume crops. For other crops, that are more distant from *M. truncatula*, as lupin, bean and peanut, these gene markers will serve as useful anchors for comparative genetics.

Genomic tools were specifically developed for pea. For example, a high throughput method making use of microarrays has been developed to rapidly characterize 2561 pea accessions. The construction of a pea BAC library has been completed corresponding to 10-11 genome-equivalents, and an efficient screening system was developed. There is no doubt that this BAC library will be invaluable for the development of pea and related grain legumes genomics.

WP5.1 Sequencing the *Medicago truncatula* genome

M. truncatula is a model organism for the study of legume biology. As such it represents a key node in the legume family, providing a platform for understanding essential aspects of legume development, as well as providing a genomics infrastructure that can be translated into the crop legumes, that possess larger or more complex genomes. It is essential to know the genome sequence of model organisms in order to facilitate research in these species and to provide the genomic infrastructure necessary for translational genomics. This workpackage has focused on deciphering the genome sequence of *M. truncatula*.

Sequencing *M. truncatula* has been an international effort with 4 sequencing centres involved: the University of Oklahoma and the J. Craig Venter Institute in the USA, Genoscope in France and the Welcome Trust Sanger Institute in the UK. The sequencing project has been supported and coordinated through efforts at the University of Minnesota, INRA-CNRS in Toulouse, Wageningen University in the Netherlands and the John Innes Centre in Norwich.

Considering that *M. truncatula* is a reference legume it was decided to generate the best quality genome sequence possible and this is achieved by a BAC-by-BAC sequencing approach. Such an approach requires a physical map of *M. truncatula* and this had already been generated by Doug Cook's group at the University of California, Davis. This physical map constituted many BAC contigs some of which were anchored to the *M. truncatula* genetic map. The Noble Foundation in the USA provided funds to the University of Oklahoma to initiate the *M. truncatula* genome sequencing. This resulted in approximately 1000 BACs being sequenced with many of the contigs possessing sequenced BACs and this represented the starting point of the International *Medicago* sequencing project.

The *M. truncatula* genome is approximately 500Mb. However, the genome structure is rather simple: euchromatic chromosome arms, with heterochromatin rich centromeres and telomeres. The sequencing initiative decided to focus on the gene rich chromosome arms that had been estimated to cover approximately 200Mb. In this way the majority of the gene space of *M. truncatula* could be deciphered by sequencing less than half the total genome. To facilitate this approach the University o Wageningen used cytogenetics to identify BACs that represented the borders of the centromeres and telomeres. These BACs provide the end points for the sequencing project.

Chromosomes were allocated to the different sequencing centres, with chromosomes 3 and 5 being sequenced at Sanger and Genoscope as part of GLIP. The BACs that had been sequenced at the University of Oklahoma were used as seed points to start the sequencing work. To improve BAC selection the ends of BACs from a number of BAC libraries were sequenced. These BAC end sequences, along with the physical map allowed the selection of the best BACs for sequencing. New BACs were selected from the seed points, and once these BACs were sequenced, additional BACs were selected. In this way the

contigs in the physical map were sequenced to completion. At the end of the contigs BACs were further selected using either the BAC end sequence database, or if this did not provide new BACs by hybridising to filters of BAC libraries.

The initial intent was to sequence 200Mb of the *M. truncatula* genome. However, as genome sequence became available it became apparent that there had been an underestimation of the euchromatin size. We now believe the euchromatim to cover approximately 300-320Mb and therefore considerably more sequence is necessary to complete this genome. The international project has sequenced 223Mb of the *M. truncatula* genome with Genoscope contributing 29Mb and Sanger contributing 36Mb. The international project is still ongoing and we anticipate the completion of this genome at the end of 2008.

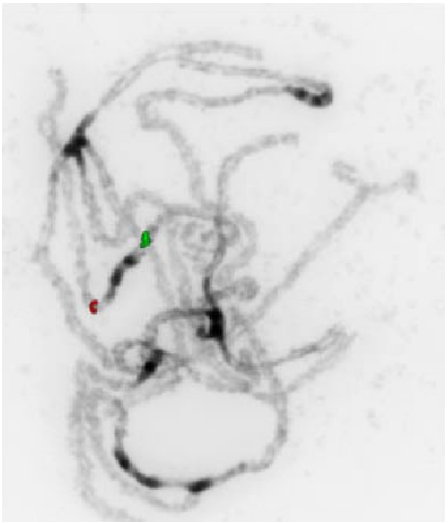


Figure 28
Chromosomes of *M. truncatula* hybridised with BACs that define the centromere/euchromatin border of chromosome 2. Note the densely packaged heterochromatin is restricted to the centromere.

WP5.2 Mutagenesis and reverse genetics

The aim of this WP was to develop three reverse genetic platforms for *Medicago truncatula* and one for Pea, based on i) a *Medicago Tnt1* mutagenized population, ii) a *Medicago* fast neutron mutagenized population and iii) EMS mutagenized populations for the *Medicago* and Pea Tilling platforms.

Tnt1 mutant collection:

Mobile genetic elements like retroelements can disrupt genes and inactivate them when inserting in genomes. The tobacco retroelement *Tnt1* is mobile in *Medicago* (d'Erfurth et al. Plant J. 2003) and can be used to construct insertion mutant collections that are suitable for forward and reverse genetics analyses. The *Medicago Tnt1* mutant collection had to be constructed by *in vitro* somatic embryogenesis during the program. Due to the choice of the ecotype used for the plant production, the objective was the creation of a collection of 5000 mutant lines representing approximately 100 000 retrotransposon inserts or 50 000 tagged genes. Mutant screening was not planned in the program but tagged genes were delivered to the scientific community after analysis of a restricted number of transposon flanking sequences.

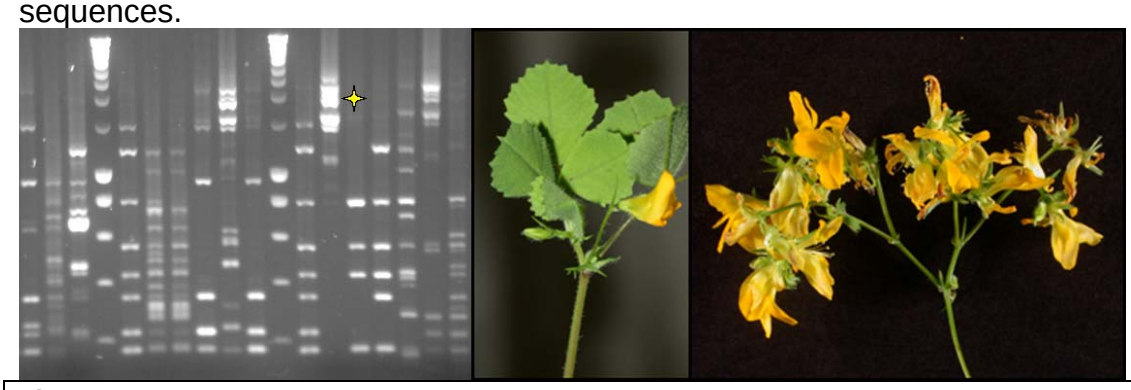


Figure 30
A, Demonstration of Tnt1 reactivation in *M. truncatula* Jemalong using a PCR transposon display technique. Each DNA band represent a transposon insertion site. The lane with an asterix is a parental line. The other lanes are regenerated individual plants. Lanes 4/11 are molecular weight markers. Lanes 6/7, 5/12 and 8/10 are sister lanes.
B, floral mutant phenotype in the wild type and *M. truncatula* MtPIM::Tnt1 mutant lines (Benlloch et al. 2006).

The 10 laboratories involved in the program were AgrobioInstitute Sofia (Bulgaria), BRC-HAS Szeged (Hungary), CNR-IRMGPF Perugia (Italy), CNRS Gif/Yvette (France), CSIC Valencia (Spain), INRA Dijon (France), INRA-CNRS (Toulouse), University of Ghent –VIB Ghent (Belgium), University of York (UK), VUB Brussel (Belgium).

In addition to the collaborative effort necessary for the construction of the collection, this work has allowed the development of new regeneration protocols for *M. truncatula* that will be very useful for the legume community. It has also given the opportunity to these 10 laboratories to develop the regeneration technology for *Medicago* in their own laboratory. Finally the work

has allowed the discovery of an endogenous mobile retroelement (*MERE*) in Medicago that can also be used in this collection for gene tagging.
The most important outcome of this work is the delivery to the scientific community of a tagged mutant collection that will be further developed and coordinated with the work done at the Noble Foundation USA (Tadege et al. Plant J. 2008). Most importantly, massive transposon flanking sequence (FSTs) sequencing is planned for both collections and will allow Web-based identification of insertion mutants for most *Medicago* genes. The first FST sequences are now available at http://bioinfo.noble.org/mt_insertion/, together with the FST generated at the Noble Foundation.

Fast neutron mutant collection:

This platform was developed at the JIC Norwich (UK) based on the concept that fast neutrons induce deletions in genomes. These deletions inactivate genes and by analyzing large mutagenised populations, deletions in target gene can be identified. The name Deletion TILLING (De-TILLING) has been adopted to describe the population structure and the detection strategies developed around this Fast neutron mutagenised population.

The PCR based screening technology is done on three dimensional pools of genomic DNAs prepared from 156 000 M2 plants representing 31 000 M1 mutagenized plants. The power of this pooling technique allows the entire population to be screened for the presence of a deletion mutant using only 52 PCRs. Further to the 156,000 M2 mutant populations developed at the JIC, an exchange has occurred with a similar population developed at the Samuel Roberts Nobel Foundation (USA). This exchange would result in a total population of over 250,000 M2 plants.

Integration of the FNB (De-TILLING) and TILLING screening for *Medicago* offers now the opportunity to streamline these activities and deliver null mutants not identified in TILLING screens (http://jicgenomelab.co.uk/).

Medicago TILLING platform:

The TILLING technique allows the detection of point mutations in any gene. We have applied this method to *Medicago truncatula*, in order to profit from the wealth of information and resources available for this species including a genome sequencing project. Using large EMS mutant collections, we are able to identify an allelic series of mutants of the gene of interest.
The platform developed during the GLIP program uses biological material prepared at INRA Dijon (France). For TILLING, the EMS mutant collection is screened using a combination of the PCR technique and the detection of DNA mismatches (the mutation) by an endonuclease. The mutant detection was done both at INRA Dijon (http://www2.dijon.inra.fr/urleg/) and at the JIC Norwich (http://jicgenomelab.co.uk/).

Mutants for 72 genes involved in a wide variety of traits were identified during the GLIP program and the seeds were delivered to laboratories of the GLIP consortium. In addition, screens have been carried out for laboratories outside the GLIP, and mutants supplied. Mutants that could not be isolated in the TILLING population could be recovered in the De-TILLING population.

Pea TILLING platform:

To develop a high throughput forward and reverse genetics tool in pea, the INRA labs at Dijon and Evry have constructed a reference EMS-mutant population and developed a database, UTILLdb, which contains phenotypic as well as sequence information on mutant genes. For the phenotyping, we have scored 4817 mutant lines for phenotypic alterations using 107 sub-categories of phenotypes. In TILLING screens we have searched for mutations in 30 genes and identified more then 600 alleles. In order to manage and integrate the expanding data from both the phenotype recordings and TILLING target genes, we implemented the database UTILLdb. UTILLdb was developed under a relational database system, interconnecting 4 main modules: lines, phenotype categories, sequences and mutations. Two main types of data are accessible, the morphological phenotypes of mutants and the sequences of tilled genes and corresponding alleles, when available. UTILLdb may be searched using a sequence, through a BLAST tool or for a phenotypic feature, by keyword search. The outcome of the search is shown as a table of results that displays the phenotype of each line, with associated pictures and mutated sequence if existing. Thus, the user could ask whether lines that share mutations in a specific gene share the same phenotypes and vice versa. As we expect a more detailed phenotypic characterization of the TILLING mutants, as they are analysed by UTILLdb users, UTILLdb was designed so that the passport data of the mutant lines can be extended or modified as needed. UTILLdb is publicly accessible through a web interface. A link is implemented to facilitate seed ordering. UTILLdb serves also as an entry point for users wishing to have their favourite gene tilled on the Caméor TILLING platform. Results from those screens as well as the phenotypes of the mutants identified will be implemented in UTILLdb. By opening this tool to the community (http://urgv.evry.inra.fr/UTILLdb), we hope to fulfil the expectations of both crop breeders and scientists who are using pea as their model of study in Europe.

WP5.3 Expression Profiling

The major objective of this Work Package was to establish central expression profiling platforms suitable for exploring the transcriptome of the model legume *Medicago truncatula* and the grain legume crop *Pisum sativum*. Considering that commercial expression profiling tools, e.g. Affymetrix GeneChips, were not available for any legume species at the beginning of the GLIP, we first had to develop microarray tools in the frame of our collaborative project. These microarrays were based on gene-specific 70mer oligonucleotide probes representing all *M. truncatula* and *P. sativum* transcript sequences that were publicly available. Together, our Mt16kOLI1Plus and Ps6kOLI1 microarrays represent more than 13.000 *M. truncatula* and 5.000 *P. sativum* unigenes, respectively. Whereas Mt16kOLI1Plus microarrays were suitable for whole-plant gene expression profiling, Ps6kOLI1 microarrays primarily relied on expressed sequences from seeds and thus were of prime interest for studying the biology of reproductive tissues. In the course of the project, more than 1,000 microarrays have been printed at the central GLIP microarray facility located at the Center for Biotechnology (Bielefeld University). This centralized production of microarrays (Figure 31) on the one hand allowed to achieve uniform quality standards throughout the lifetime of the project and on the other hand facilitated an up-to-date development of array-related information, such as regular updates of probe annotations and the deposition of arraylayouts in public databases, e.g. the EBI ArrayExpress database.

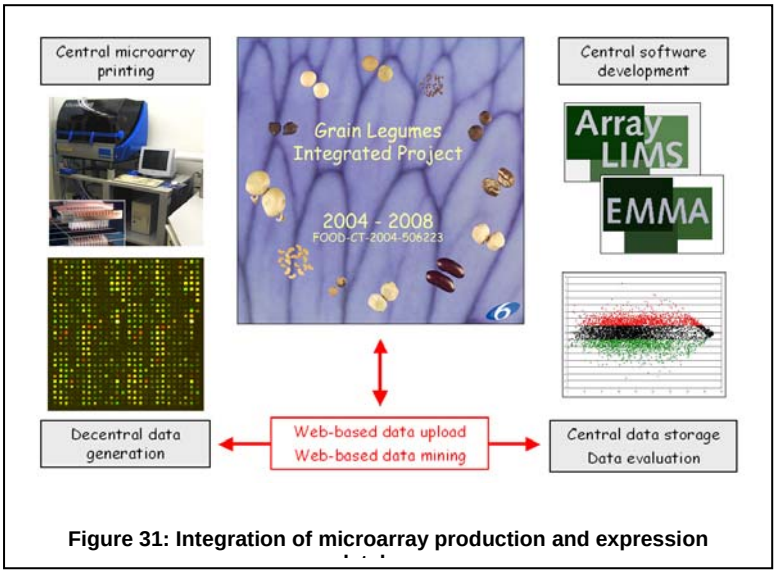


Figure 31: Integration of microarray production and expression

In addition to providing global transcriptome profiling tools, a real-time RT-PCR platform specifically designed to monitor the expression of *M. truncatula* transcriptional regulators was developed at the Max-Planck-Institute for Molecular Plant Physiology in Golm, in collaboration with Work Package 3.2. To set up this tool, the *M. truncatula* genome sequence was mined for those genes encoding putative transcription factors. Until the end of the GLIP, validated gene-specific primers for 1084 TF genes were available, with a continuing objective to increase their number periodically, as more *M. truncatula* genome sequence become available.

Based on the Mt16kOLI1Plus and Ps6kOLI1 microarrays as well as the real-time RT-PCR platform, a range of expression profiling experiments has been performed either by GLIP partners in their own facilities or in the central

facilities for microarray hybridizations and real-time RT-PCR at Bielefeld University and the MPI Golm, respectively. In general, these studies involved the transcriptome profiling of wild type and mutant lines of the model legume *M. truncatula* and the grain legume *P. sativum*, investigating biological conditions that were of specific relevance for the biological processes studied in GLIP modules 3 and 4. Thus, the expression data obtained addressed different time courses of seed development (in wild-type plants, mutants, and transgenic lines) and a range of interactions with aerial pathogens (e.g. *Erysiphe pisi*, *Aschochyta* spec., *Phoma* spec., blue-green aphids), root pathogens (e.g. *Aphanomyces euteiches*), root parasites (e.g. *Orobanche crenata*) as well as symbiotic interactions with nitrogen-fixing rhizobia or arbuscular mycorrhizal fungi.

In collaboration with Work Package 6.1, a transcriptomics webserver has been developed that allowed to upload microarray expression data generated in the frame of the GLIP to a central ArrayLIMS data repository. From this data repository, expression data have been imported into the EMMA, an ontology-based software that allows to group microarray hybridizations into experiments addressing a particular biological question, e.g. the development of legume seeds or the response to different pathogens. Until the end of the GLIP, 40 experiments containing more than 550 microarray hybridizations have been stored in the EMMA database (Figure 31). From this database, gene expression information can easily be retrieved using standard web browsers. In particular, the quick mining of expression data across all conditions profiled in GLIP is possible for members of the GLIP consortium, that way integrating the expression information obtained in different laboratories (Figure 31 and 32). The EMMA database is interfaced to an automated annotation software that provides not only updated annotation but that also facilitates the projection of gene expression information onto metabolic pathways, allowing to draw conclusions on metabolic effects that accompany developmental changes or that characterize particular mutants. Combined with the phenotypical analysis of mutants generated in the frame of Work Package 5.2., the gene expression information generated in the GLIP can be useful to select candidate genes for legume breeding (Figure 32)

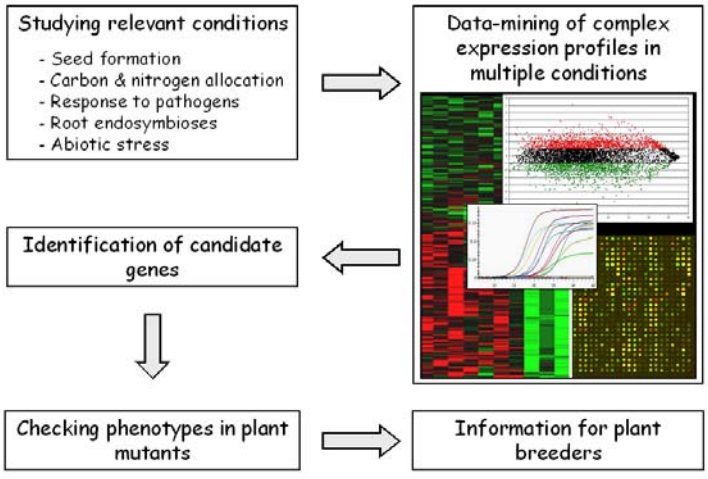


Figure 31: Strategy to identify candidate genes for legume breeding.

Due to the fact that both the generation and the interpretation of transcriptome profiles involves advanced knowledge, one important aspect of our activities was the training of young researchers. In the course of the project, two advanced transcriptomics courses have thus been held at Bielefeld University, primarily addressing the bioinformatics aspects involved in gene expression profiling. In addition, several members from different GLIP groups have individually been instructed to perform experiments related to microarray hybridizations and real-time RT-PCR experiments at Bielefeld University or at the MPI Golm, respectively.

In conclusion, the introduction of a central microarray platform in the GLIP has allowed us to set up an efficient mode of distributing standardized microarray tools in sufficient quantities to the different groups involved in the project and also to other European, Canadian and Australian laboratories via the GL-TTP. Similarly, the existence of a central real-time RT-PCR platform was beneficial to efficiently organize the profiling of transcription factor gene expression in GLIP, as a step to identify important regulators of legume development. The existence of a central database for microarray data is certainly unique in the legume community, and current work involves the development of webservices that allow an even easier mining of expression information also for non-expert users. The Truncatulix data warehouse, designed as an easy-to-use public repository for *M. truncatula* expression data, is already publicly available (https://lily.cebitec.uni-bielefeld.de/truncatulix/app) and will be developed further to host GLIP expression data being ready to be released into the public domain.

WP5.4 Crop & Comparative Genomics

A general strategy in this project relied on comparative genomics in crop species. The basic idea is that we should focus a lot of effort on characterizing one genome in great detail and work out how the other genomes relate to this. The more closely related two genomes are the more likely they are to have the same genes in the same relative order. The question we had to answer was how distantly related could two legumes be for this relationship to be useful in practice. Genome comparisons were performed by genetic mapping of gene targeted intron spanning PCR based markers. (PCR primer sequences are at http://bioweb.abc.hu/cgi-mt/pisprim/pisprim.pl)

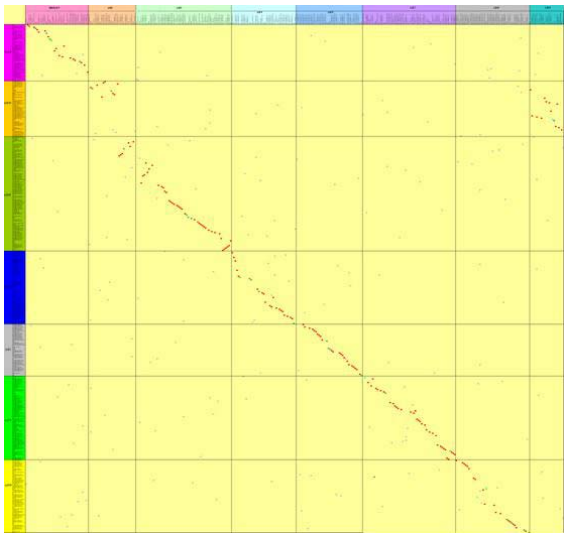
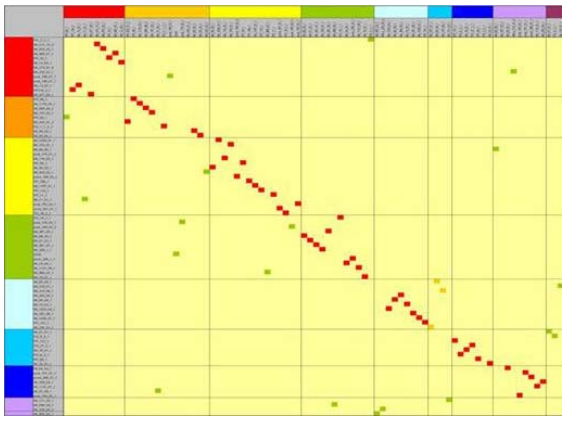


Figure 33 The dotted line in the figure above compares gene order in pea and *M. truncatula,* while the figure below compares this genome to that of chickpea



Amazingly it seems as though the *Medicago truncatula* genome sequence is useful for all of the species we have studied. It follows from this that any legume genome sequence is useful throughout this group of plants. Strictly speaking we know this is the case just for those legumes with pea-like flowers, and we do not know about this for the other legumes (mainly tropical tree species).

Pea is the most widely grown legume field crop in Europe and its genome is very large, about the same size as the human genome and about ten times the size of the *Medicago truncatula* genome. This is one of the main reasons why pea has not been the major focus for genomic studies. One objective in this project was to prepare a permanent and comprehensive collection of large DNA fragments of pea. To this end a Bacterial Artificial Chromosome library with approximately 10X genome coverage and insert sizes in the range of 95-180 kbp.

Germplasm collections are a major resource for breeders and genetics. The *Pisum* germplasm collection at the John Innes Centre (http://www.jic.ac.uk/germplas/pisum/)) has been studied in some detail and we wanted to understand how other collections throughout Europe compared to this one. To that end we studied the pattern of allelic variation at 31 genetic loci in a total of 2561 accessions and the results suggest that genetic diversity in both groups of germplasm was largely overlapping.

Module 6 Bioinformatics (WP6.1)

Objectives

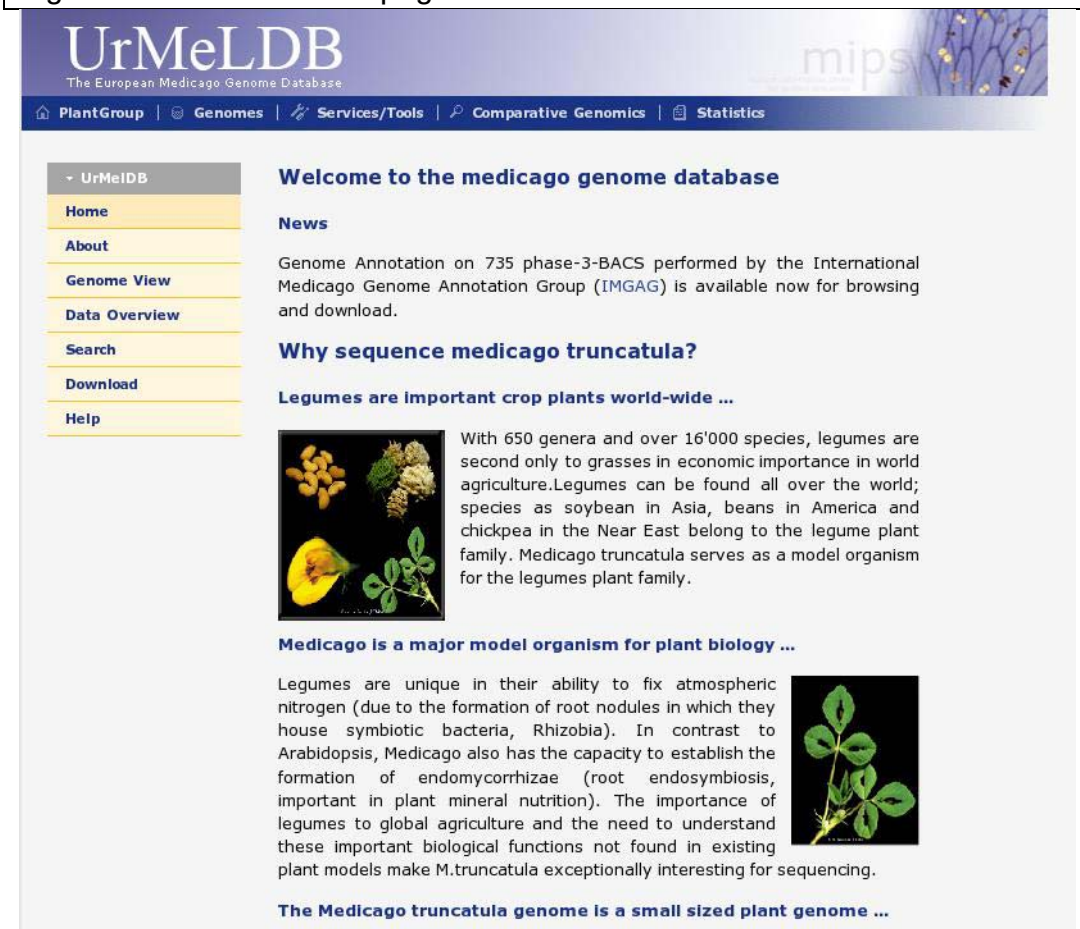
Driven by the large amounts of –omics type data have been generated in the frame of GLIP, the complexity of data and biological questions, the bioinformatics work Module covered a wide spectrum of activities. The achievements in the Module include the development, implementation and population of variety of different type of databases used to organise, store and communicate data generated as well as the development of different types of bioinformatic analytical tools to address genome scale and comparative analysis in grain legumes.

The main achievements within the Module are the development of adequate databases and population of these databases for:

- the development of a European Medicago and Legume Database genome database (*UrmelDB).* Aim was to deliver a high quality genome sequence annotation as an important information basis for the interpretation of experimental data. An important component of the work was to communicate the analysis data through adequate means, that is databases and suitable interfaces.

http://mips.gsf.de/proj/plant/jsf/medi/

Figure 34 UrMeLDB web page at MIPS



- the development of a comprehensive transcriptomics information and analytical system to allow storage and analysis of expression data generated and to assist in easy web-based data-mining of expression profiles from *Medicago truncatula.* Along with the implementation of an adequate ArrayLIMS system for this purpose, a web-based EMMA transcriptomics web server has been developed. Our purpose was to display transcriptomic data generated in the IP and allow the scientific community to analyse the data by customizable input parameters and selections of underlying datasets.

- the development, adaptation and application of bioinformatic analytical tools for comparative analysis and display of legume genomics and germplasm data. The Grain Legumes project involved the development of comparative mapping data for several species. Gene-based maps have been developed for pea, faba bean, chickpea, lentil, phaseolus, lupin and groundnut, with genes anchored to the *Medicago truncatula* sequence. The objectives of this task has been to develop a comparative mapping database, the GERMINATE database system (http://bioinf.scri.ac.uk/germinate). Where the aim is to store and communicate the comparative mapping data into the database and to provide an intuitive graphical interfaces to the data.

- embedding of the bioinformatics efforts of the IP within the international bioinformatics framework. Legume centric and legume genome centric research activities take place beyond GLIP activities. However knowledge and data generated in theses activities are complementary and should thus enrich the GLIP data matrix by collaboration with relevant groups and consortia outside the IP. GLIP bioinformatic activities were successfully coordinated with external efforts and thus profits from highly synergistic activities.

- Finally new bioinformatic approaches have been developed to analyse the evolutionary dynamics of legume germplasm (and plant germplasm in general). This encompassed a new theoretical approach to estimate genetic diversity within germplasm collections and the development of a new software tool which allows analysis of patterns of genetic diversity within germplasm collections of grain legumes to be carried out.

Module 8 Dissemination and Transfer
Throughout the project, Module 8 facilitated the interface of GLIP with the outside and strengthened networking and the dissemination of knowledge among scientists and stakeholders related to legume production and uses in Europe and worldwide.

WP8.1 Dissemination of Knowledge
During GLIP, the web (see: www.grainlegumes.com) together with hard-copy publishing has enabled continuous dissemination of information and several events such as specific scientific workshops or information days contribute to a strong networking with different scientists in Europe and worldwide and different stakeholders in the EU.

Disseminating information
In addition to the web sites:

Figure 35. Web page screen shots.

publishing of scientific articles (in peer reviewed journals) or thematic syntheses related to GLIP activities especially in *Grain Legumes* magazine targeting a wide community of scientists, economic players and decision makers such as the progress in GLIP integrated activities presented in the *Grain Legumes* magazine.

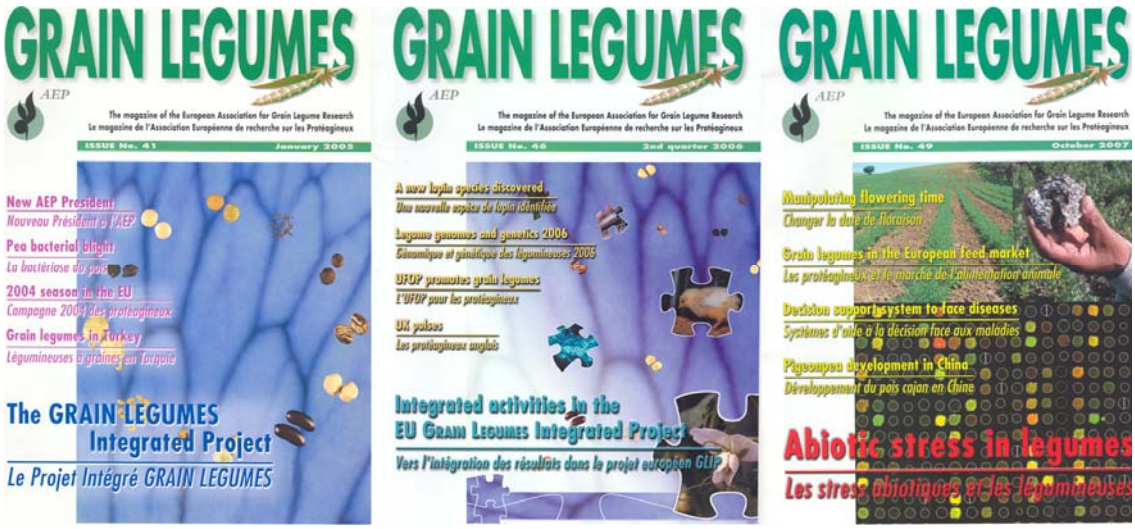


Figure 36. Grain Legumes Magazine issues 41, 46 and 49 which feature GLIP

Module 8 has aided the GLIP interface with other EU and international groups working on legumes notably with GL-PRO, Eufaba and Eurocrop. Links with international activities: GCP, ICLGG3, Medicago Sequencing Consortium, IFLRC-IV and IFLRC-V Steering Committee and the Phaseomics consortium have been maintained both through individual participants and by the coordinating activities of AEP.

Specific workshops for enhanced integration of legume science have been held such as: 'Ascochyta 2006', the GL-Pro and GLIP Dissemination event in Brussels, 3-4 May 2006 and the GL-TTP workshop in Paris in 2007. There have also been several specific dissemination events at the national and international level, notably the Sixth European Grain Legumes Conference in November 2007, details of all of these can be found on the AEP web site (www.grainlegumes.com).

WP8.2 Transfer and exploitation of results

This WP created a new legal entity as a project participant to act as an agent for external organisations within GLIP. The idea was that this would be a self sustaining organisation that provided and negotiated access to relevant technologies or intellectual property. This platform was created in 2006 and attracted much interest and an extended membership that remains in control of the activities of the platform. While the finances of this entity did not fully permit our initial ambitious goals the platform did provide access as anticipated and its continuation post GLIP marks its success.

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