Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes

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Keywords: metabolomics; mutagenesis; plant transformation; proteomics; tissue culture; transcriptomics

Abstract

Biotic and abiotic stresses cause significant yield losses in legumes and can significantly affect their productivity. Biotechnology tools such as marker-assisted breeding, tissue culture, *in vitro* mutagenesis and genetic transformation can contribute to solve or reduce some of these constraints. However, only limited success has been achieved so far. The emergence of “omic” technologies and the establishment of model legume plants such as *Medicago truncatula* and *Lotus japonicus* are promising strategies for understanding the molecular genetic basis of stress resistance, which is an important bottleneck for molecular breeding. Understanding the mechanisms that regulate the expression of stress-related genes is a fundamental issue in plant biology and will be necessary for the genetic improvement of legumes. In this review, we describe the current status of biotechnology approaches in relation to biotic and abiotic stresses in legumes and how these useful tools could be used to improve resistance to important constraints affecting legume crops.

Introduction

Legumes are among the most important crops worldwide, having major impacts on agriculture, the environment, animal/human nutrition and health (Graham & Vance 2003). Legumes can interact symbiotically with specific soil-borne bacteria, the rhizobia, which allow the plant to fix atmospheric nitrogen and may help to protect them against some fungal pathogens (Chakraborty et al. 2003). As such, they constitute a significant source of nitrogen and consequently play an essential role in both the structure of ecosystems and sustainable agriculture, worldwide. These symbiotic interactions have strongly driven the investigation and application of biotechnology tools for legumes. Nevertheless, a number of biotic (fungi, bacteria, nematodes, viruses, parasitic plants, insects) and abiotic (drought, freezing, salinity, waterlogging) stresses are severely affecting the yield of these crops.

The adaptability and productivity of legumes are limited by major abiotic stresses including drought, heat, frost, chilling, waterlogging, salinity and mineral toxicities. Although the type and the severity depend on the specific crop location, abiotic stresses can result in crop damages as high as those caused by biotic stresses. Furthermore, crops under abiotic stress are usually more susceptible to weeds, insects and diseases, which increase considerably the losses (Reddy et al. 2004). An additional factor relevant to the legumes is the response of the symbiotic nitrogen-fixing bacteria to stresses. Application of biotechnology approaches to these crops can contribute efficiently to solve or reduce these problems.

Successful application of biotechnology to biotic/abiotic constraints facing legume crops will require both a good biological knowledge of the target species and the mechanisms underlying resistance/tolerance to these stresses. The large genome size and the polyploidy of some legumes have hampered this goal, but in order to solve some of these problems two species, *Medicago truncatula* and *Lotus japonicus*, have emerged as model plants to investigate the genetic of nodulation and other important processes such as resistance or

tolerance to stresses. Their respective small and diploid genomes, autogamous nature, short generation times, and prolific seed production were important characteristics for these choices (Cook 1999; Handberg & Stougaard 1992). Since then, powerful genetic and genomic tools have been developed, such as genome sequencing (Kato et al. 2003), isolation of Expressed Sequence Tags (ESTs; Asamizu et al. 2004; Kulikova et al. 2001) and the establishment of genetic and physical maps for each model species (Pedrosa et al. 2002; Thoquet et al. 2002). The increasing wealth of genetic and genomic data and the high degree of synteny between legume genomes (Kalo et al. 2004; Stracke et al. 2004), make these two species valuable models for the molecular genetic study of the biotic and abiotic constraints that hamper legume crop yield.

Much of the research on plant stress responses in this area has been conducted with Arabidopsis as a model system. Substantial similarities between the defence responses of Arabidopsis and legumes exist, however, there are also significant differences (Anderson et al. 2005). Thus, it is necessary to increase our understanding of the specific aspects of the defence/stress responses in legumes in order to solve some of the major constraints facing these crops. In this review, relevant advances in marker-assisted breeding, tissue culture, genetic transformation, and gene expression, including large-scale approaches and functional analyses are presented and discussed as a way to overcome biotic and abiotic stresses in legumes.

Major biotic and abiotic stress targets for improvement in legumes

Legume production is greatly constrained by numerous biotic and abiotic stresses. Many of the diseases, pest and abiotic stresses are common to all legume crops; however, their incidence and importance vary according to the legume crop, management practices and regions.

Biotic stresses

The major biotic stresses affecting legumes are fungal diseases although insects, nematodes, viruses, bacteria and parasitic weeds can drastically decrease legume production. Weeds are also a problem for many legume crops but will not be covered here.

The relative importance of aerial fungal diseases and their effect on yield varies among years and cropping regions. However, some of them affect large areas in all the countries where legumes are cultivated and cause considerable losses in quality and quantity. Foliar diseases caused by biotrophic pathogens, such as rusts, downy mildews and powdery mildews, are major limiting factors in legume production and the most important of these are present in all areas where legumes are cultivated (Sillero et al. 2005, this issue). Several rust species can infect grain and forage legumes, most of them belonging to the genus *Uromyces*, such as *U. appendiculatus* on common bean, *U. ciceris-arietini* on chickpea, *U. pisi* on pea, *U. striatus* alfalfa, *U. viciae-fabae* on faba bean, lentil and common vetch and *U. vignae* on cowpea. Also rust species belonging to other genera can be major problems on legumes such as *Phakopsora pachyrhizi* and *P. meibomiae* on soybean or *Puccinia arachidis* on groundnut (Rubiales et al. 2002). Asian rust (*Phakopsora pachyrhizi*) is a severe disease that causes important yield losses in soybean and is spreading rapidly around the world (Carmona et al. 2005; du Preez et al. 2005; Pivonia & Yang 2004). Lack of natural sources of resistance (Ramteke et al. 2004) makes this disease a good candidate to be solved using biotechnology. Normally, legume rust epidemics begin late in the season, when pod filling has started, so yield components are only slightly little affected by the infection and losses are usually low. However, when the infection starts early in the season severe epidemics can occur (Rashid & Bernier 1991).

Powdery mildew is an important fungal disease in several legumes caused by *Erysiphe pisi* (Sillero et al., 2005, this issue). Powdery mildew of pea has a worldwide distribution being particularly important in climates with warm, dry days and cool nights, adversely affecting yield and quality. Severe infection may cause 25-50% yield losses (Warkentin et al. 1996). Downy mildew, caused by *Peronospora viciae* occurs in most places where the crops are grown, but is most frequent and severe in cool, maritime climates (Sillero et al., 2005, this issue).

The major necrotrophic fungal diseases are ascochyta blight on various grain legumes, chocolate spot on faba bean and anthracnose of lupin and lentil (Tivoli et al. 2005, this issue). Ascochyta blight, caused by *Ascochyta rabiei*, is the most important fungi disease of chickpea. It affects above-ground parts of the plants causing 100% yield loss in some situations (Nene & Reddy, 1987). Botrytis gray mould caused by *Botrytis cinerea* is of lesser importance in chickpea but also a widespread foliar disease problem. The common foliar diseases on faba bean are Ascochyta blight and Chocolate spot. Ascochyta blight, caused by the fungus *Ascochyta fabae*, is distributed world-wide (Gaunt, 1983). Yield losses of about 40% are common, but losses can be as high as 90% in susceptible cultivars (Hanounik, 1980), particularly under wet and cool weather conditions. Chocolate spot, caused by *Botrytis fabae*, is a destructive leaf disease of faba bean that can reduce yields by more than 60 % (Hanounik, 1981), particularly in humid regions. Ascochyta blight is considered the most important necrotrophic foliar disease on pea worldwide (Bretag & Ramsey, 2001). It is caused by three related fungal species, commonly referred to as the Ascochyta complex: *Ascochyta pisi*, *Ascochyta pinodes* (teleomorph *Mycosphaerella pinodes*) and *Phoma medicaginis***.** The major foliar necrotrophic pathogens on lupins are anthracnose, caused by *Colletotrichum lupine*, followed by Brown spot, caused by *Pleiochaeta setosa* and Phomopsis, caused by *Diaporthe toxica* (Sweetingham et al. 1998). Ascochyta blight of lentils, caused by *Ascochyta lentis* has

been reported worldwide in most lentil producing countries (Bayaa & Erskine, 1998). Anthractonse of lentils, caused by *Colletotrichum truncatum* is a common and important pathogen on lentil in Canada (Anderson et al., 2000), althought of little importance in several countries in Asia and Africa (Bayaa & Erskine 1998).

There are several soil-borne diseases that are common among legume crops (Infantino et al. 2005, this issue). Most of these attack the seedling stage of the crop and are referred to as damping-off diseases. For example, damping-off, generally caused by either *Rhizoctonia solani* or *Pythium* spp., can result in up to 80% of plant death (Denman et al. 1995; Wang et al. 2003). Fusarium root-rot (caused by *Fusarium* spp.) can also cause severe seedling losses especially in common bean and lentils (Hamwieh et al. 2005; Schneider et al. 2001). In most growing areas of the world, Fusarium wilt (caused by *F. oxysporum*) is a major constraint in the production of pulse crops, chickpea (Navas-Cortés et al. 2000; Nene & Reddy 1987) and lentil (Bayaa et al. 1997) in particular. The disease affects seedlings and adult plants where it causes leaf chlorosis, wilting and death. Other important soil-borne diseases such as southern stem rot (*Sclerotium rolfsii*) and the white mold (*Sclerotinia sclerotiorum*) can cause both seedling and pod rots in warmer and cool weather respectively (Kolkman & Kelly 2003).

A number of parasitic plants have become weeds, posing severe constraints to major crops including grain legumes (Rubiales et al. 2005, this issue). *Orobanche crenata* is an important problem in most cool season legumes in the Mediterranean basin and Middle East. Yield loss can be severe with complete loss of crops in severe cases. *O. aegyptiaca* is of importance in the Middle East and Asia. *O. foetida* is widely distributed in natural habitats in the Western Mediterranean area parasitizing wild herbaceous leguminous plants, but is however considered an important agricultural parasite in the faba bean in Beja region of Tunisia. *O. minor* is of economic importance on clover that is grown for seed and has recently become a problem on red clover in Oregon, USA (Rubiales 2001; Rubiales et al. 2005). *Striga*

gesnerioides and *Alectra vogelii* cause considerable yield reduction of grain legume crops, particularly cowpea, throughout semi-arid areas of sub-Saharan Africa (Parker & Riches 1993).

Viruses cause yield losses for most legume crops. For example, Bean Common Mosaic Virus (BCMV) and its close relative, Bean Common Mosaic Necrotic Virus (BCMNV) are the most widespread and frequent viruses of common bean leading to significant losses. In addition, over the past two decades, Bean Golden Mosaic Virus (BGMV) has been considered the most important yield limiting disease for bean production in parts of Central America and the lowlands of the Caribbean, with yield losses between 10 and 100% (Coyne et al. 2003).

Insects are another important biotic stress faced by many legume crops. They cause important damages both through direct feeding, as vectors or by providing infection sites for pathogens (Edwards & Singh 2005, this issue). Examples of important insect pests in grain legumes include aphids like *Aphis glycine*, pod borers such as *Helicoverpa armigera* and *H. punctigera* in cool season legumes (Yoshida et al. 1997) and weevils such as *Apion godmani* and *Zabrodes subfasciatus* in warm season legumes (Garza et al. 1996; Romero-Andreas et al. 1986).

Abiotic stresses

Abiotic stress is a broad term, which includes multiple stresses such as heat, chilling, excessive light, drought, waterlogging, wounding, ozone exposure, UV-B irradiation, osmotic shock and salinity. It has been estimated that only 10% of arable land can be classified under the non-stress category, which implies that crops grown on the other 90% of arable lands experience one or more environmental stresses. Some of these stresses like drought, extreme temperature, and high salinity dramatically limit crop productivity. The prediction is that water deficits will continue to be the major abiotic factor likely to affect crop yields globally

(Sharma & Lavanya 2002). Moreover in many legumes such as peanut (*Arachis hypogaea*), Brazil nuts (*Bertholletia excelsa*) and faba bean (*Vicia faba*), this stress is particularly important because pre-harvest aflatoxin contamination is a common occurrence (Arrus et al. 2005; Mahmoud & Abdalla 1994) that can be reduced in drought tolerant lines (Holbrook et al. 1994). On the other hand, waterlogging due to a combination of unfavourable weather conditions and suboptimal soil and irrigation techniques can result in severe yield losses (Dennis et al. 2000). Waterlogging limits the oxygen diffusion of the soil and as a consequence nitrification is replaced as the most important N-transforming process, by denitrification and/or nitrate ammonification (Laanbroek 1990). Furthermore, under waterlogging stress, plant potassium, sodium, iron, and manganese uptake are limited and crops become more susceptible to diseases. For instance, waterlogging peas are more susceptible to *M. pinodes* (McDonald & Dean 1996).

Soil salinity affects total nitrogen uptake and soil nitrogen contribution (van Hoorn et al. 2001), leading to yield reduction. It is also expected that with the decrease in the ozone layer, UV exposure will become an important stress for cropping system (Chimphango et al. 2003).

Several of the abiotic stresses associated with legume crops can also directly affect symbiotic interactions and therefore limit legume growth. *Sinorhizobium meliloti* shows pH-sensitivity below pH 6 reducing *Medicago sativa* development, while *Mesorhizobium loti* is tolerant up to pH 4, facilitating the growth of *Lotus glaber* in more acid soils (Correa et al. 2001). Deficiencies and toxicities of micronutrients are also an important constraint of legume crops. Limitation of growth due to boron toxicity or deficiency has been described for instance in pea or faba bean (Dwivedi et al. 1992; Poulain & Almohammad 1995). In some cases such as soybean, the deficiency or toxicity is more critical for root nodulation than for the direct growth of the plant (Rahman et al. 1999).

To face the threat represented by these stresses several genetic improvement strategies are available, from classical breeding to a more direct physiological-genetic approach. However, only with an understanding of the mechanisms underlying a specific stress, will the later strategy be feasible. In general for the stresses mentioned above, low yields in developing countries are primarily due to a lack of effective disease management practices, particularly the availability of disease-resistant cultivars. Moreover excessive and often inappropriate fungicide usage in many situations, such as occurs with the control of bean rust, can contribute to higher input costs, human health problems and contamination of water supplies and the environment. In this context, biotechnology is a powerful tool that has potential to contribute to sustainable agriculture. Biotechnology approaches such as marker-assisted breeding, tissues cultures, *in vitro* mutagenesis, and genetic transformation can contribute to speed up classical breeding and overcome major problems such as lack of natural sources of resistance and sexual incompatibility.

The fact that many of these stresses such as the pathogens, *Colletotrichum trifollii*, (Torregrosa et al. 2004), *Aphanomyces euteiches* (Nyamsuren et al. 2003), *Uromyces striatus* (Rubiales & Moral 2004), nematodes (Koltai et al. 2001), *Phytophthora megasperma* f. sp. *medicaginis*, *Fusarium* spp., *Ascochyta* spp. (Salzer et al. 2000) and the parasitic plant *O. crenata* (Rodríguez-Conde et al. 2004) also affects the legume model *M. truncatula*, will help increase our understanding of the underlying molecular and genetics basis of resistance, and consequently increase the potential for biotechnology to overcome these stresses in the major legume crops.

Biotechnology tools

Molecular marker-assisted breeding

The use of genetic and genomic analysis to help identify DNA regions tightly linked to agronomic traits in crops, the so-called molecular markers, can facilitate breeding strategies for crop improvement. The use of molecular markers for the indirect selection of improved crops speeds up the selection process by alleviating time-consuming approaches direct screening under greenhouse and field conditions. Molecular markers are particularly useful when targeting characters controlled by several genes. The potential to map different Quantitative Trait Loci (QTL) contributing to an agronomical trait and to identify linked molecular markers opens up the possibility to transfer simultaneously several QTLs and to pyramid QTLs for several agronomical traits in one improved cultivar.

Numerous molecular marker-related techniques have been used in legumes in relation to biotic and abiotic stresses. Random Amplified Polymorphism (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) and derivatives have been reported both for biotic (Ouedraogo et al. 2002; Román et al. 2002) and abiotic (Kassem et al. 2004; Lee et al. 2004) stresses. As a result, genetic maps for many species were established in which potential resistance and/or tolerance loci or QTLs have been located (Table 1 and 2). This improved the knowledge of the genetic control of specific resistance and/or tolerance in many legumes by providing information on the number, chromosomal location and individual or interactive effects of the QTLs involved. More importantly, these technologies have identified specific molecular markers that may be used in breeding programs through Marker-Assisted Selection (MAS) to enhance stress tolerance.

However, the application of molecular markers in breeding programs requires preliminary studies to identify and validate potential markers. In this process, the following factors have to be considered: a) level of polymorphism existing between parental lines, b) unclear expression of some markers inherent to the marker class used, c) false-positive

markers, d) discrepancy between the presence of the marker and target gene, which requires testing the gene with conventional screening and e) presence of multiple genes scattered over several linkage groups (Yu et al. 2004).

Although the use of MAS may be helpful for crop improvement, its practical application in legumes for the genetic improvement of resistance or tolerance to stress have been limited, being mainly hampered by lack of investment and the genetic complexity of most stress-related traits. There are some exceptions where MAS has already facilitated breeding efforts for several legume crops against important biotic stress. For instance MAS was successfully used for the breeding of resistant soybean to cyst nematode (Diers 2004), of resistant pinto bean to common bacterial blight (Mutlu et al. 2005) and of resistant narrow-leafed lupin (*Lupinus angustifolius* L.) to phomopsis stem blight (Yang et al. 2002) and anthracnose (Yang et al. 2004). Moreover, when resistance is conferred by single genes and/or easily overcame by new pathogens races, the gene pyramiding strategy facilitated by MAS can be an efficient method. Breeding for abiotic stress is much more complicated due to the complexity of the traits involved. Nevertheless, Schneider et al. (1997) showed that MAS may be useful to select drought tolerant common bean.

Gene pyramiding assisted by MAS

Breeding durable resistance to biotic and/or abiotic stresses is a major task for plant breeders and pyramiding different resistance or tolerance genes into a genotype is one way of achieving this. There are numerous examples of introgression and pyramiding of favourable alleles and QTLs in legumes. However, only in a few cases has MAS been used to assist in gene pyramiding to overcome stresses. Most relevant work has been carried out in common bean breeding for rust and anthracnose resistance (Faleiro et al. 2004). There are RAPD markers linked to the 11 genes (*Ur-1* to *Ur-11*) conferring rust resistance and these markers

are being used to incorporate and pyramid rust resistance into common bean cultivars, and/or to combine rust resistance with resistance to other diseases, such as BCMV, BGMV, common bacterial blight, and/or anthracnose (Singh 2001; Stavely 2000). Similarly, molecular markers linked to the majority of genes conferring anthracnose resistance (*Co-1* to *Co-10*) have been described, thereby providing the opportunity to pyramid them in a resistant cultivar through MAS (Kelly & Vallejo 2004). In the quest for resistant cultivars to multiple stresses, combining several biotechnological approaches such as transgenesis or mutagenesis and MAS, to pyramid multiple resistance genes appears as a powerful strategy. Such an approach was recently achieved in soybean to manage insect resistance, resulting in the enhancement of resistance levels to corn earworm (*Helicoverpa zea*) and soybean looper (*Pseudoplusia includens*) in eight soybean lines in which two major insect-resistance QTLs and a synthetic Bt gene (*cry1Ac*) were combined (Walker et al. 2004).

The general knowledge of abiotic stress QTLs in legume is still at an early stage so that gene pyramiding has not been applied yet. Nevertheless, advances achieved in non-legume crops such as tomato, in which many salt stress tolerance QTL have been identified and validated, open the possibility to transfer all of them to obtain a single improved cultivar (Foolad 2004).

Thus, legume cultivars having appropriate combinations of resistance and/or tolerance genes to biotic and abiotic stresses, achieved through gene pyramiding, could provide durable resistance, and MAS can be a valuable tool to guide and identify the pyramiding of these genes. Nevertheless, it is important to validate the results with resistance or tolerance tests, due to the possibility of gene mutations, background effects, recombinants and adverse interactions among resistance genes that can occur during breeding programs and influence the expected phenotype. In addition, combining molecular markers with other technologies may improve the efficiency of MAS. Recently, the combination of MAS with biolistic

transformation was used in rice to achieve multiple resistance against blast and bacterial blight disease (Narayanan et al. 2004). Moreover, the use of the information generated by gene expression experiments may help to improve MAS (Figure 1). Gene expression analysis helps to increase the understanding of the molecular basis of stress resistance in plants. Generation of markers based on genes with altered expression patterns in response to stresses, could result in more effective and targeted MAS. Some of these genes are described in the gene expression section and may be good candidates for futures MAS studies in legumes.

Tissue culture

In grain legumes, tissue culture has been repeatedly described as difficult. Regeneration from both organogenesis and embryogenesis has been recalcitrant in this plant group (Anand et al. 2001; Chandra & Pental 2003). This recalcitrance towards *in vitro* regeneration is a major constraint in transgenic plant production for many legumes, since advances in molecular genetics, e.g. gene over-expression, gene suppression, promoter analysis and T-DNA tagging, require efficient transformation systems (Somers et al. 2003). Efficient tissue culture is therefore a vital step, required for both the validation and exploitation of data generated by these powerful molecular tools. Implementation of robust protocols for regeneration is therefore a necessary condition for both genetic transformation and other tissue-culture derived techniques to generate genetic diversity such as somaclonal variation, *in vitro* mutagenesis, doubled haploids culture, and wide hybridization.

Somaclonal variation and in vitro *mutagenesis*

A little explored strategy for legume breeding is the capacity of tissue culture to generate genetic variations. Tissue culture generates a wide range of genetic variation in plants, which can be incorporated in plant breeding programmes (Jain 2001). It is well-known

that somaclonal variation involving callus cultivation and somatic embryogenesis has the capacity to generate genetic variation (Larkin & Scowcroft 1981). The possibility of producing agronomically-useful somaclones via organogenesis and somatic embryogenesis has already been reported in pea (Griga et al. 1995) and pigeonpea (Chintapalli et al. 1997). These variations are not desirable for some applications such as genetic transformation or massive micropropagation, but can be useful for breeding. These techniques, separately or combined with chemical or physical mutagenesis, generate diversity, which is a major breeding goal.

In vitro mutagenesis strategies such as treatment with Ethyl-Methane-Sulphonate (EMS), fast neutron radiation and insertional mutagenesis have been applied in plant breeding. These methods induce point mutations, deletions, or insertions, respectively and have been useful in breeding for biotic (Bhagwat & Duncan 1998; Kowalski & Cassells 1999) and abiotic (Fuller & Eed 2003; Khan et al. 2001) stress in non-legume crops. In legumes most effort has occurred with nitrogen fixation (Sagan et al. 1994), and mutants with resistance or tolerance to stresses have not been described. Efforts in this area have been hampered by the recalcitrance of legumes to regeneration and the low efficiency of finding the desired phenotypes. Nevertheless, the improvement of regeneration protocols for many legumes and the performance of induced mutant crop cultivars indicate that *in vitro* mutagenesis can play an important role in legume breeding. Indeed, combining mutagenesis techniques with MAS through TILLING as described below will make mutagenesis more attractive and applicable for legume improvement. The major difficulty with these techniques is the high quantity of individuals required to find the desired trait. Nevertheless, by using *in vitro* selection systems this disadvantage can be minimized.

In vitro *selection*

In vitro selection has been used for both biotic and abiotic stress. The best studied biotic stresses have been fungal diseases, using toxins or filtrate culture as selective agents (Svabova & Lebeda 2005). *In vitro* selection resulted in the isolation of resistant lines in carnation to *Fusarium oxysporum* f.sp *dianthi* (Thakur et al. 2002), in strawberry to *Alternaria alternata* (Takahashi et al. 1992), and in wheat to *Fusarium graminearum* (Ahmed et al. 1996). Salinity is the main abiotic stress that has been addressed by *in vitro* selection (Flowers 2004; Zair et al. 2003), although applications to other stresses, such as zinc tolerance, have also been reported (Samantaray et al. 1999).

Currently, these techniques are considered to be an important complement to classical breeding methods (Svabova & Lebeda 2005), although they have not been sufficiently explored in legumes. *In vitro* selection in legumes has been carried out mainly in alfalfa (*Medicago sativa*) for selection to *C. trifolii* (Cucuzza & Kao 1986), *F. oxysporum* (Cvikrova et al. 1992) and *Verticillium albo-atrum* (Koike & Nanbu 1997). Theses studies showed the feasibility of *in vitro* selection in legumes, although no resistant lines were reported. This system can also be coupled to other approaches in addition to somaclonal variation. Putative stress-resistant lines derived from both conventional breeding and transgenic approaches could be screened using *in vitro* selection. This is particularly attractive for some abiotic stresses, where appropriate screening methods are unavailable or have low efficiency. Although the advantages of the recent high-throughput technologies, coupled with genetic transformation, are emerging as attractive approaches, somaclonal variation and *in vitro* mutagenesis following by *in vitro* selection offers an alternative way for breeding.

Double haploids and wide hybridization

Doubled haploid (DH) technology refers to the use of the microspore or anther cultures to obtain haploid embryos. This technology offers breeders a tool for the rapid

production of homozygous lines. These homozygous lines can be multiplied and released as cultivars, or used as recombinant inbred lines for molecular mapping and/or in breeding programs (Martinez et al. 2002). An efficient DH production technology can greatly reduce the time and cost of cultivar development (Liu et al. 2002a) and some stress-improved non-legume varieties have been produced with this technology (Qian et al. 2000). Since DH is a tissue culture dependent-technique, legumes have been generally recalcitrant and as far we know no commercial legume varieties have been produced using this technology. However, some advances have been achieved in alfalfa (Zagorska et al. 1997), *Lupinus* spp. (Bayliss et al. 2004; Ormerod & Caligari 1994) and soybean (Cardoso et al. 2004; Rodrigues et al. 2005). The importance of this approach for plant breeding in Europe, has led to the COST action 851 "Gametic Cells and Molecular Breeding for Crop Improvement" (http://www.scri.sari.ac.uk/assoc/cost851) led by Brian P. Forster (Scottish Crop Research Institute) which includes some legumes. Successful application of DH technology to legumes in the near future would be a major achievement. However as not all homozygous lines are of interest, the coupling of DH and MAS technology will be more efficient to select individuals carrying desirable traits.

Wide hybridization depends on various factors, and according to Sharma (1995) can be as wide as one can make them. Stress-related characters available in wild germplasms could be introgressed into economic target species through improved wide hybridization techniques such as embryo rescue and protoplast fusion. Efficient embryo rescue has allowed the production of interspecific hybrids in legumes such as faba bean (*Vicia faba* x *V. narbonensis*; Lazaridou et al. 1993), grass pea (*Lathyrus odoratus* x *L. belinensis*; Hammett et al. 1994), and pigeonpea (*Cajanus cajan* x *C. platycarpus*; Mallikarjuna & Moss 1995). Stress responses have not been assessed in these hybrids. However, the potential of this technique has been demonstrated in non-legumes crops where successful breeding for stress tolerance

has been reported (Bradshaw et al. 1997; Tonguc & Griffiths 2004). Protoplast fusion also has potential applications for crop improvement by overcoming sexual incompatibility or reproductive barriers, and by generating novel combinations of nuclear and/or cytoplasmic genomes (Liu et al. 2005). Intergeneric somatic hybrid plants between sexually incompatible legume species have been reported in alfalfa (*Medicago sativa* x *Onobrychis viciifolia* and *Medicago sativa* x *Lotus corniculatus*; Kaimori et al. 1998; Li et al. 1993) and pea (*Pisum sativum* x *Lathyrus sativus*; Durieu & Ochatt 2000). Additionally, somatic hybrids between legumes and non-legume species has been developed, for instance the hybrids generated from *Vicia faba* x *Helianthus annus* (Schnabl et al. 1998) and *Lotus corniculatus* x *Oriza sativa* (Nakajo et al. 1994). Interestingly, some regenerated plants from the hybrid calli of the latter fusion were tolerant to low temperatures and low sunlight intensity. Despite the potential of this technique, limited efforts have been applied to overcome stresses in legumes. Nevertheless, the successful transference of resistance or tolerance achieved in non-legumes crops to biotic (Hansen & Earle 1995) and abiotic (Arumugam et al. 2002; Brewer et al. 1999; Yue et al. 2001) stresses together with advances in tissue culture in legumes, should encourage legume breeders to exploit somatic hybrids. For an excellent overview of advances on intergeneric somatic hybridization and its application to crop genetic improvement see Liu et al. (2005).

Genetic transformation

Crop improvement through genetic engineering has become a reality (Dunwell 2000). It is now possible to transform many grain legumes (Chandra & Pental 2003; Somers et al. 2003) although in some cases the rate of recovery of transgenic lines is still low. The use of *Agrobacterium tumefaciens* as a vector for legume transformation was an important breakthrough. Both micro-particle bombardment (Gulati et al. 2002; Li et al. 2004) and *A.*

tumefaciens (De Clercq et al. 2002; Li et al. 2004) have been used for DNA delivery into either embryogenic or organogenic cultures. Transformation has been generally based on infection by *A. tumefaciens*, although *A. rhizogenes* is also used for transformation of some species to produce composite plants with hairy roots or hairy root cultures (Boisson-Dernier et al. 2001; Stiller et al. 1997; Wu & VanEtten 2004). The inserted DNA can be either a specific gene with a specific biochemical function, a regulatory gene that controls a network of other genes, or multiple genes to generate long-term durable resistance. In this review we will describe only examples related to biotic or abiotic stress. For a more comprehensive review about transformation and gene technology in legumes, other references should be consulted (Chandra & Pental 2003; Popelka et al. 2004; Somers et al. 2003).

A number of legume cultivars have been transformed in order to enhance the resistance to biotic stresses. Resistance to insects using *Bacillus thuringiensis* genes (Walker et al. 2000) and viruses using pathogen-derived resistance (Aragão et al. 2002), along with the introduction of constitutively expressed genes encoding pathogenesis-related (PR) proteins or phytoalexins (He & Dixon 2000; Samac et al. 2004) have been reported in legumes (Table 3).

Abiotic stresses generally involve perturbation of various cellular functions and activation of complex metabolic pathways, and are conferred by polygenic traits (Kassem et al. 2004; Lee et al. 2004; Popelka et al. 2004). This complexity together with the lack of good sources of natural tolerance makes this an area that is not readily amenable for conventional breeding strategies. In plants there is a poor understanding of most abiotic stress responses. Thus, the successful use of genetic transformation requires a better physiological and molecular understanding of these stresses. Recent progress achieved in non-legume plants supports the potential use of transgenic approaches to produce tolerant lines (Jiang et al. 2004; Kasuga et al. 1999; Shou et al. 2004; Sivamani et al. 2000; Umezawa et al. 2004). For instance, the use of transgenic, mutagenic and genetic approaches strongly improved the

understanding of the genetic and molecular mechanisms of salinity tolerance in plant, and this will help develop crops, including legumes, with improved tolerance (reviewed in Apse & Blumwald 2002; Foolad 2004; Hasegawa et al. 2000). As a result, it was found that over-expression of a single-gene controlling vacuolar or plasma membrane Na+/H+ antiport protein, in transgenic Arabidopsis, tomato and rape seed provided them with a high level of salt tolerance under greenhouse conditions (Apse et al. 1999; Shi et al. 2003; Zhang & Blumwald 2001; Zhang et al. 2001). Similarly, manipulating expression of pea DNA Helicase45 or the glyoxalate pathways confers high salinity tolerance in tobacco (Sanan-Mishra et al. 2005; Singla-Pareek et al. 2003). Although transgenic plants are yet to be examined for salt-tolerance in the field, the recent genetic advances suggest there are good prospects for developing transgenic legumes with enhanced salt tolerance in the near future (Foolad 2004; Sharma & Lavanya 2002). On the other hand the increase of tolerance to aluminium and cyanamide toxicity in transgenic alfalfa (Morphew et al. 2004) and soybean (Zhang et al. 2005) demonstrates the potential of this approach in legumes. For a more exhaustive review of the application of transgenesis to overcome abiotic stresses in plants, see Sharma & Lavanya (2002).

As described above genetic transformation is an attractive approach to overcome stresses in legumes. Nevertheless, as far we know the only transgenic legumes commercially used for biotic stress is the soybean line carrying the insect resistance gene *cryA* from *B. thuringiensis* (Babu et al. 2003). This low number has been influenced by technical (regeneration recalcitrance of most legumes), social (public concern issues) and political (lower rate of investment in legume crops compared to other crops such as rice, wheat and maize) reasons. However, the advances in legume transformation protocols and the increasing interest in legumes as high protein content food should see an increase in the production of genetically modified legumes.

Gene expression

As already mentioned, the efficiency of both MAS and transgenic approaches will be improved by using the information from gene expression studies. Understanding the mechanisms employed by plants to defend themselves against stresses and a more complete knowledge about the genes involved, will allow a more precise use of MAS and transgenics. Sequence information, while valuable and a necessary starting point, is insufficient to answer questions concerning gene function, regulatory networks and the biochemical pathways activated in response to stresses. To address these questions, more comprehensive approaches, including quantitative and qualitative analyses of gene expression products, are necessary at the transcriptomic, proteomic, and metabolomic levels.

Transcriptomics

An important step in the control of stress responses in plants is the transcriptional activation or repression of genes (Chen et al. 2002). Thus, identification of differentially expressed genes is particularly important to understand stresses response in plants. To achieve this objective, tools such as microarrays (Schena et al. 1995), Suppression Subtractive Hybridization library (Diatchenko et al. 1996), Serial Analyse of Gene Expression (Velculescu et al. 1995) and quantitative measurement of transcription factor (TF) expression patterns have been developed in addition to older techniques such as Northern blotting.

In legumes, gene expression patterns following biotic stresses have been more extensively studied than those following abiotic stresses. Large-scale analyses of gene expression patterns in response to pathogens have revealed the differential expression of large numbers of genes. Known defence gene families are usually expressed differentially in these studies independent of the specific legume-pathogen interaction being investigated. Among these genes, phytoalexins such as medicarpin (Blount et al. 1992; He & Dixon 2000),

PR-proteins including PR-10, chitinases, glucanases (Salles et al. 2002) and lipoxigenases (*Lox* genes) have been frequently detected (Cho & Muehlbauer 2004; Torregrosa et al. 2004).

The coupling of these powerful large-scale gene expression profiling methods with recombinant inbred lines or near isogenic lines in legumes that differ in susceptibility/resistance to key pathogen and pests will greatly facilitate a more comprehensive understanding of genes involved in the defence response of legumes to specific biotic stresses. For more details on legume plant-pathogen interactions or legume-pest interactions, see Torregrosa et al. 2005 and Edwards & Singh 2005, respectively, in this issue.

In respect to abiotic stress, gene expression analyses have been mainly based on studies with cloned genes (Singh et al. 2004). Other work has shown that transcriptomic tools are also a good option for legume breeding to environmental stresses. Using a modified c-DNA-AFLP technique in soybean, 140 differentially expressed c-DNA fragments were obtained by comparing control and iso-osmotic treated plants. Some of the responsive genes encoded ion transporters, transcription factors (TFs) and redox enzymes (Umezawa et al. 2002). Suppression subtractive hybridization screening was carried out in *Retama raetam*, a C-3 drought tolerant legume. This study revealed that dormancy, key to the survival of many species in arid environments, was followed by accumulation of transcripts encoding a PR-10 like protein, a low temperature-inducible dehydrin and a WRKY transcription factor (Pnueli et al. 2002). Similarly, 43 drought-responsive mRNA transcripts were reported to be differentially expressed in peanut following water stress (Jain et al. 2001).

Much more extensive expression studies have been performed in Arabidopsis, and the resulting knowledge can also be used in legumes through comparative genomics. For example, Ishitani et al. (2004), selected 100-200 genes from the Arabidopsis database, and showed that at least three *DREB*-like genes, thought to be key transcriptional regulators of

drought and/or cold tolerance were present in common bean. Many other expression studies in Arabidopsis have highlighted the involvement of TFs in stress responses and this has encouraged researchers working with other plants to focus on these proteins.

Transcription factors

Transcription factors are proteins that play an important role in controlling the expression of genes in most biochemical pathways including the response to stress (Eulgem 2005; Kasuga et al. 1999). Genomics studies over the last few years have identified numerous TFs (mainly in Arabidopsis) and revealed a high degree of complexity and overlap in the transcriptional regulation of gene expression in response to many stresses (Shinozaki & Yamaguchi-Shinozaki 2000). The understanding of the role of TF may open new avenues for improving resistance or tolerance to stresses (Singh et al. 2002). While large-scale analysis of TFs have been primarily done in Arabidopsis (see review by Eulgem, 2005), a platform for the study of TFs in *M. truncatula* has been developed by Dr. M. Udvardi (Max Planck Institute for Plant Physiology, Golm, Germany).

An interesting family of TFs for stress responses in plants is the ethylene-responsive-element-binding factors (ERF) of which over 60 members have been described in *M. truncatula* (Anderson et al. 2005); and the closely related DREB/CREB proteins (Singh et al. 2002; Yamaguchi-Shinozaki & Shinozaki 2005). Members of ERF family are responsive to cold, drought, pathogen infection, or wounding. The WRKY family, involved in the regulation of plant stress-response genes such as receptor protein kinases (Asai et al. 2002; Robatzek & Somssich 2002), and bZIP family members that regulate PR-1 and Glutathione *S*-Tranferase genes (Chen & Singh 1999; Fan & Dong 2002; Lebel et al. 1998), or cold/dehydration genes (Yamaguchi-Shinozaki & Shinozaki 2005) are other important stress-

responsive TF families. A Krüppel-like TF (*Mtzpt2-1*) involved in salt tolerance has also been described in *M. truncatula* (Merchan et al. 2003).

A given TF can mediate the response to various stresses (Eulgem 2005; Yamaguchi-Shinozaki & Shinozaki 2005). This characteristic makes the TFs especially attractive for genetic transformation, because a single TF gene can result in resistance or tolerance to various stresses. Following this principle, over-expression of a TF that regulates an ABA-responsive gene conferred multiple stress tolerance in rice (Kim et al. 2004). However, different TFs are also known to respond to the same stress with different but overlapping kinetics (Onate-Sanchez & Singh 2002). On the other hand, attempts to knockout specific TFs have often not resulted in any obvious phenotypes, perhaps due to overlapping function. Thus, use of TFs for genetic improvement requires a comprehensive knowledge of their biological functions.

Proteomics

In parallel to the accumulation of a wealth of genomic and transcriptomic data, recent technological developments have allowed the establishment of valuable methods for quantitative and qualitative protein profiling (Cánovas et al. 2004). This approach is very important in evaluating stress-responses, because mRNA levels do not always correlate with protein accumulation (Gygi et al. 1999). Indeed, large differences in protein turnover and post-translational modifications may lead to large variations between transcriptomic and proteomic data. Thus, protein studies are needed to provide information on their levels and activities (Zivy & de Vienne 2000). To this purpose, proteomic-based techniques that allow large-scale protein profiling are powerful tools for the identification of proteins involved in stress-responses in plants (Gygi & Aebersold 2000).

Extensive studies have evaluated changes in protein levels in plant tissues in response to stresses (Cánovas et al. 2004; Kim et al. 2003). Unfortunately, these studies have been mainly focused on non-legume species, such as Arabidopsis and rice (Cánovas et al. 2004), and only recently have been enlarged to include some legumes (Jorrín et al. 2005, this issue). As a result only a handful of studies have been carried out in legumes, although in the next few years there should be a significant increase in the number of legume species and stresses analysed. So far, pea has been more intensively studied, with the analysis of induced protein expression in roots in response to salt (Kav et al. 2004), to cadmium stress (Repetto et al. 2003) and to infection by the parasitic plant *Orobanche crenata* (Castillejo et al. 2004). In addition, proteomic approaches have been applied to *M. truncatula*, lentils, lupin, common bean, cowpea and soybean to identify proteins involved in the response to different stresses (Colditz et al. 2004; Fecht-Christoffers et al. 2003; Kav et al. 2004; Mithofer et al. 2002; Pinheiro et al. 2005; Repetto et al. 2003). Interestingly, many of the induced proteins from these different stresses were common or belonged to overlapping pathways. For example, members of the PR-10, the phytoalexin biosynthesis enzyme and the peroxidase families were identified in different studies. These observations highlight a potential role of these genes for resistance or tolerance to stress in legumes. Although further studies are needed to determine their exact function in stress-response, these genes could be promising candidates for genetic transformation and/or MAS approaches (Figure 1).

Metabolomics

Transcriptomic and proteomic data are important steps in deciphering a complex biological process, but they are still insufficient to understand them fully since most biological processes are ultimately mediated by cell metabolites. Alternative mRNA splicing, protein turn-over rates and post-translational modifications that modulate protein activity

imply that changes in the transcriptome or proteome do not always correspond to alterations in the cell metabolome (Sumner et al. 2003). Therefore, the only way to the complete understanding of both gene function and molecular events controlling complex plant processes is to analyse in parallel the transcriptome, the proteome and the metabolome in an integrative manner (Dixon 2001). In legumes, this kind of approach has been taken in *M. truncatula* suspension cells to various stimuli (Bell et al. 2001), and with the characterization of metabolic changes during the nitrogen-fixing symbiotic interaction in *L. japonicus* (Colebatch et al. 2004; Desbrosses et al. 2005; Rispail 2005).

Although large-scale, comprehensive metabolomic studies are difficult, a number of targeted analyses have been performed to assess the involvement of subsets of metabolites in various stresses. Most studies on plant stress responses focused on flavonoid and isoflavonoid phytoalexins. Accumulation of medicarpin, pisatin, glyceolin or sativan has been frequently observed in response to pathogen infection and elicitor treatment in alfalfa, pea, soybean and *L. japonicus* respectively (Baldridge et al. 1998; BorejszaWysocki et al. 1997; Lozovaya et al. 2004; Saunders & O'Neill 2004; Shimada et al. 2000). Interestingly, phytoalexin accumulation was also observed after copper or mercury stress (Mithofer et al. 2004). The involvement of these compounds as key defence metabolites has been nicely proven by the modification of resistance levels to *Phoma medicaginis* and *Nectria haematococca* in alfalfa and pea cultivars respectively (He & Dixon 2000; Wu & VanEtten 2004).

Other metabolite classes have been described as potential defence or signal molecules. For instance, *L. japonicus* leaves have been shown to emit many volatile terpenoids in response to spider mites. The exact roles of these molecules are still unclear, but they are believed to play a role as attractants for natural predators of herbivorous insects and as systemic signal defence inducers for neighbouring plants (Ozawa et al. 2000). The possibility to attract predatory mites by modulation of sesquiterpene synthesis through transgenesis as

recently demonstrated in Arabidopsis may have important repercussions in alternative insect-damage protection (Kappers et al. 2005). The triterpene saponins also appear to be important in defence reactions as they accumulate in response to insect attacks in alfalfa (Agrell et al. 2004) and are well-known for their allellopathic, antimicrobial and anti-insect activities (Dixon & Sumner 2003). These targeted studies highlight the importance of secondary metabolites in stress resistance in legumes.

While the preliminary results from combining metabolic approaches with transgenics indicates the potential of increasing intrinsic stress resistance levels in legume crops and strengthens the potential role of biotechnology in crop improvement (He & Dixon 2000; Wu & VanEtten 2004), it must be remembered that most metabolic pathways are interconnected in highly complex networks. Thus, modulating one metabolic pathway may have negative impacts on another, leading to concomitant deleterious traits in the modified crop. Large-scale metabolic analyses are therefore necessary to observe the metabolic networks important for plant growth and development under a range of environmental conditions.

Large-scale analysis by using different “omics” technologies are providing extensive data sets that will help identify potential candidate genes for an increase in intrinsic resistance and/or tolerance levels in important legume crops. Identification of these candidate genes, may allow their direct application in crop improvement through MAS, or genetic engineering. However, in most cases, the roles of these candidate genes remain unknown and it will be important to carry out functional studies as a preliminary step towards their use in genetic improvement.

Functional analysis

To date the completion of the Arabidopsis and rice genomes have been achieved and the genome of some legumes (*M. truncatula*, *L. japonicus*) together with other plant

sequencing projects is underway. The traditional pursuit of a gene starting with a phenotype (forward genetics), has given way to the opposite situation where the gene sequences are known but not their functions. The challenge is now to decipher the function of the thousands of genes identified by genome projects, and reverse genetics methodologies are key tools in this endeavour (Gilchrist & Haughn 2005).

The ability to knockout genes or suppress their expression are powerful tools to determine the function of a gene. This can be done by anti-sense RNA suppression, targeted gene replacement, insertional mutagenesis, gene silencing and Targeted Induced Local Lesion In Genome (TILLING) approaches. Anti-sense RNA suppression requires considerable effort for any given target gene before even knowing whether it will be successful (McCallum et al. 2000) and targeted gene replacement i.e. via homologous recombination has not yet been reproducibly achieved for higher plants.

Collections of random T-DNA (over 225,000 independent *Agrobacterium* T-DNA insertions) or transposable element insertion mutants are currently available for the Arabidopsis community (Alonso 2003). While such a collection does not exist yet in legumes, insertional mutagenesis has been successfully used. For example, in *L. japonicus* identification of regulatory components of nodule induction have been achieved by characterizing a transposon-tagged mutant, *nin*, arrested at the stage of bacterial recognition (Schauser et al. 1999). However, although collections of T-DNA mutants may be very useful, they produce a limited range of allele types and do not always produce null alleles (Rispail 2005; Webb et al. 2000). Recently the use of the tobacco retrotrasposon Tnt1 has been successfully applied for large-scale insertional mutagenesis in *M. truncatula* and promises to be a useful tool for functional genomic (Tadege et al. 2005).

The term RNA silencing has been adopted to describe phenomena such as post-transcriptional gene silencing in plants, quelling in fungi and RNA interference in animals

(Baulcombe 2004). Researchers have developed different RNA silencing strategies as tools for selective knockout of targeted genes. Despite the successes of this technique in several species, RNA silencing has several drawbacks, i.e. phenotypic instability in later generations (Hannon 2002) and the requirement for a reliable plant transformation system. RNA silencing is believed to be a natural plant defence against viruses. Following this principle, another technique, Virus-Induced Gene Silencing (VIGS), has been developed to suppress plant gene expression through infection with virus vectors that harbour a target region of the host gene (Baulcombe 2004; Britt & May 2003). There are vectors with the ability to support VIGS in plants (Dalmay et al. 2000; Liu et al. 2002b). Nevertheless, in legumes, VIGS has not been extensively used. Interestingly, there are recent reports that a VIGS vector based on the Pea Early Browning Virus (PEBV) can also be used successfully in legume species (Constantin et al. 2004; Van den Boogaart et al. 2004).These findings together with the advances in legume genomic should increase the use of VIGS as a functional genomic tool in the near future.

The limitations of RNA silencing or insertational mutagenesis, previously stated, can be overcome by TILLING. This technique combines chemical mutagenesis with a powerful screening method for potential mutations (Gilchrist & Haughn 2005; Henikoff et al. 2004; McCallum et al. 2000). The generation of phenotypic variants without introducing foreign DNA in the plant makes TILLING very suitable not only for functional analysis, but also for agricultural applications. The TILLING facility for a *L. japonicus* collection of mutants is available from the Parniske group at the John Innes Centre (Perry et al. 2003). This facility has been validated by identification of 15 homozygous mutants representing six different alleles of SYMRK, an important symbiotic gene (Perry et al. 2003). A database comprising information on individual mutant plants in their collection is also accessible at http://www.lotusjaponicus.org/finder.htm. In *M. truncatula*, TILLING programs have been set up by Professor D. Cook (U.C. Davis, USA) and by Dr. A. Kondorosi (CNRS, Gif-Sur-

Yvette, France). The advantages of TILLING are resulting in private companies, such as Anawah (http://anawah.com/programs), extending TILLING facilities to a wider variety of organisms including soybean and peanuts. The diversity of species for which this technique will be available, opens up new possibilities for legume researchers both for the functional analysis of genes previously identified by the "omic" technologies (Figure 1), as well as the generations of new varieties.

Conclusions

Over the years biotechnology has emerged as a promising tool to overcome stresses in plants, but to date, progress has been limited in legumes. The current advances in tissue-derived techniques, genetic transformation and MAS, together with the advances in powerful new 'omic' technologies offer great potential to improve this situation. Indeed, it is now possible to target almost all legume crops with a variety of biotechnological approaches for genetic improvement. As such, the more efficient regeneration protocols recently established for many legumes should encourage legume researchers to resume the use of techniques such as DH, wide hybridization and mutagenesis in breeding programmes. On the other hand, crops without appropriate regeneration protocols may also be improved by mutagenesis through TILLING. It is important to provide breeders with the broadest variety of biotechnology tools as possible since each stress-crop case has its own particularities and so would need one or a combination of specific biotechnological approach(es) to tackle them efficiently. Strategies such as resistance gene pyramiding assisted by MAS could be useful to overcome resistance breakdown by new races of *U. appendiculatus* in common bean, while pathogen-derived genes and heterologous expression of PR-genes may be a better approach to enhance resistance to viruses and polyphagous fungi, respectively.

Although the advances in biotechnology greatly facilitate legume improvement, a more comprehensive knowledge of resistance or tolerance mechanisms is required to direct breeding. Indeed, only a better understanding of the underlying mechanisms activated in response to stresses will allow an efficient application of biotechnology in sustainable agriculture. The advent of the 'omic' technologies together with the functional genomic tools is a promising approach to achieve this. Most advances in these fields have been performed in Arabidopsis which has provided us with a growing understanding of important stress pathways. Nevertheless, legumes offer a number of attractive features in their own right that are drawing researchers interested in abiotic and biotic stress responses. For example, they provide an excellent system to analyse how plants distinguish between friend and foe. They also have advantages over some of the other plant model species for specific stresses. For example, *M. truncatula* has good genetic resistance to aphids (Klingler et al. 2005), something which is lacking in Arabidopsis. Thus it is important to better characterise legume responses to stress. The establishment of the model legumes *M. truncatula* and *L. japonicus* is starting to provide applicable information for legumes. The integration of knowledge generated by the different approaches described here, should lead to more accurate and efficient breeding of key legume crops. In the case of genetic engineering, this would not only allow the targeting of transgene expression to particular conditions (e.g using stress-responsive or tissue-specific promoters), but also monitoring the effect of the transgene (e.g by proteomic and metabolomic approaches). Additionally, researchers dealing with others strategies such as MAS or even classical breeding will be able to take advantage of the results being gathered from "omic" technologies. However, the delivery of "omic" information should be done in a friendlier mode for plant breeders in order to facilitate its efficient application in genetic improvement. Overall, for biotechnology to fulfill its potential for legume breeding there needs to be good and regular communication between classical

breeders and biotechnologists to firstly, make sure that the tools of biotechnology are applied to the most pressing and appropriate problems and secondly, to ensure that pathways for delivery/uptake into breeding programs are in place.

Acknowledgment

We thank to Fundación Carolina for M. A. Dita's scholarship. E. Prats is funded by an European Marie Curie Reintegration Grant, N. Rispail by (FP5) Eufaba project. Our work in this area is supported by Spanish CICYT project AGL-2002-03248 and European Union project FP6-2002-FOOD-1-506223. K. Singh's work in this area is supported in part by the Grains Research and Development Corporation (GRDC) and the Department of Education, Science and Training (DEST) in Australia. We also apologize for not being able to include all the important references because of length limitations.

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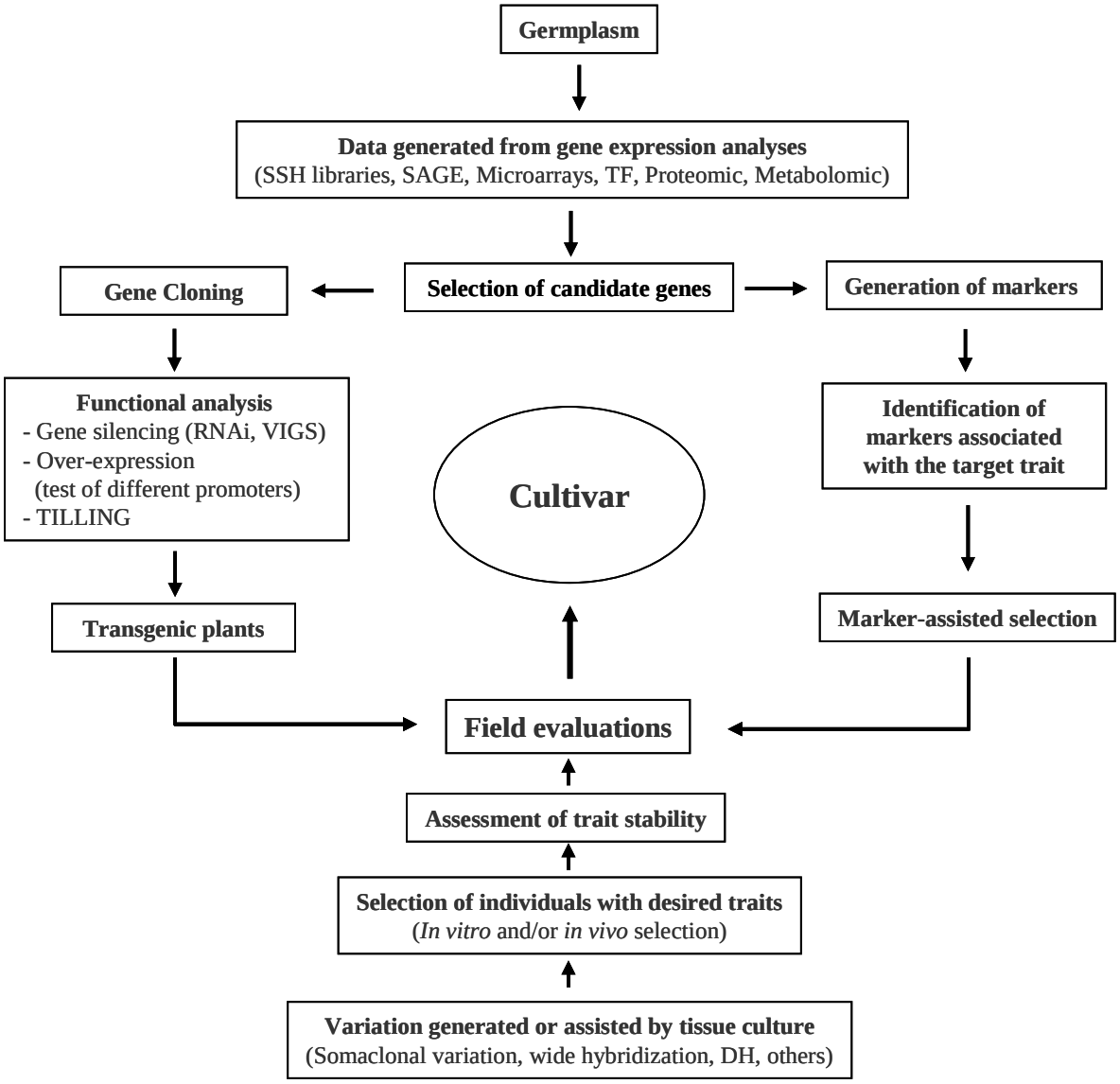

Figure 1. Integrated scheme outlining key steps for plant molecular breeding using biotechnology. VIGS: Virus Induced Gene silencing; RNAi: RNA interference; TILLING: Targeted Induced Lesion IN Genome; TF: Transcription Factors; SAGE: Serial Analyses of Gene expression; SSH: Suppression Subtractive Hybridization; DH: Doubled haploid.

Table 1: List of some QTL identified in important legume crops associated to biotic stresses

Legume	Biotic stresses	Gene(s)/QTL	Associated markers	Marker type	Referente(s)
Cicer arietinum	*Ascochyta rabiei*	*Ar*19	GA20 GA24	STMS STMS	Cho et al. (2004)
	Fusarium oxysporum f. sp. *cicer*		TR59	STMS	Cobos et al. (2005)
Lens culinaris	*Ascochyta lentis*	*ral*2 *AbR1*	$UBC227_{1290}$ and $OPD10_{870}$ *RB18 and RV01*	RAPD RAPD	Tar'an et al. (2003b)
	Colletotrichum truncatum		$OPD6_{1290}$	RAPD	Tar'an et al. (2003a)
	Fusarium oxysporum f. sp. lentis	*FW*	*OPK15900*	*RAPD*	Eujayl et al. (1998)
Lupinus angustifolius	*Diaporthe toxica*	*Phr1*	Ph258M2	SCAR	Yang et al. (2002)
	Colletotrichum gloeosporioides	*Lanr1*	AntjM1	SCAR	Yang et al. (2004)
Vicia faba	*Orobanche crenata*	*Oc1* *Oc2* *Oc3*	$OPJ13_{686}$ / $OPAC02_{730}$ $OPAC06_{342}$ / $OPN07_{849}$ $OPAA07_{807}$ / $OPAA07_{807}$	RAPD RAPD RAPD	Roman et al. (2002)
	Ascochyta fabae	*Af1* *Af2*	$OPA11_{1045}$ / $OPAB07_{1026}$ $OPE17_{1272}$ / $OPJ18_{626}$	RAPD RAPD	Roman et al. (2003)
	Uromyces viciae-fabae	*Uvf-1*	$OPI20_{900}$	RAPD	Avila et al. (2003)
Vigna unguiculata	*Striga gesnerioides*	*Rsg*1 *Rsg3* *994-Rsg*	SEACTMCAC83/85 E-AGA/M-CTA_{460} E-AAG/M-AAC_{450}	SCAR AFLP	Boukar et al. (2004) Ouedraogo et al. (2002)

RAPD: Random Amplified Polymorphism DNA; RFLP: Restriction Fragment Lengh Polimorphism; AFLP: Amplified Fragment Lengh Polimorphism; SCAR: Sequence Charactherized Amplified Region; STMS: Sequence Tagged Microsatellite Site; SSR: Simple Sequence Repeat; STS: Sequence Tagged Site; EST: Expressed Sequence Tag.

Table 1 (continuation). List of some QTLs identified in important legume crops associated to biotic stresses

Legume target	Biotic stresses	Gene(s)/QTL	Associated markers	Marker type	Reference(s)
Phaseolus vulgaris	*Xanthomonas axonopodis* pv. *phaseoli*		D1287 BNG71 BNG21	SCAR RFLP RFLP	Kelly et al. (2003)
			G19.1800; *PHVPVPK-1* PV-tttc001; BNG060	RAPD; SSR SSR; RFLP	Tar'an et al. (2001)
			BNG191	RFLP	Yu et al. (2004)
			SU91 / R7313 AI07.600 BnG154; BNG25a	SCAR RAPD RFLP; RFLP	Miklas et al. (2003)
	Fusariumsolani f. sp. *phaseoli*	*Pv*PR2 *Pv*PR1	$P7_{700}$ $S8_{500}$ $G3_{2000}$	RAPD RAPD RAPD	Schneider et al. (2001)
	Empoasca fabae *Empoasca kraemeri*		PV-atcc003 U73	SSR RFLP	Murray et al. (2004)
	Sclerotinia sclerotiorum	*fin* *Phs*	O15.1800 PR-aggctt85	RAPD SSR	Kolkman & Kelly (2003) Miklas et al. (2001)
Pisum sativum	*Orobanche crenata*	*Ocp1* *Ocp2*	P482 $OPB11_{541}$	STS RAPD	Valderrama et al. (2004)
	Erysiphe pisi	*er*	ScOPD-10_{650}	SCAR	Janila & Sharma (2004)
	Mycosphaerella pinodes		ccta2 cccc1 acct1	SSR SSR SSR	Tar'an et al. (2003b)
	Pea Seed-Borne Mosaic Virus (PSbMV)	*sbm-1* *sbm-2*	e1F4E *e1F(iso)4E*		Gao et al. (2004)

Table 2: List of major QTLs identified in important legume crops associated to abiotic stress. Avreviation of marker type are as defined for Table 1.

Legume target	Abiotic stress	Gene(s)/ QTL(s)	Associated Markers	Marker type	Reference(s)
Lens culinaris	Cold	*Frt*	$OPS16_{750}$	RAPD	Eujayl et al. (1999)
	Winter hardiness		ubc808_3_ubc807_3	SSR	Kahraman et al. (2004)
			ubc840_3	SSR	
			cs48_1	RAPD	
			ubc808_12	SSR	
			E3M3	AFLP	
Glycine max	Manganese toxicity		BARC_SATT318	SSR	Kassem et al. (2004)
			BARC_Sat092	SSR	
			BARC_SATT305	SSR	
			BARC_SATT239	SSR	
			$OE02_{1000}$	RAPD	
	Salt stress		Sat-091	SSR	Lee et al. (2004)
	Waterlogging		Sat-064	SSR	VanToai et al. (2001)
	Phosphorus deficiency	*fsw1*	L37_2I_Sat_36a	SSR	Li et al. (2005)
		fsw2 and rp1	K4-2V	RFLP	
		fsw3	B212T	EST	
		rp2	Satt114_Satt334	SSR	
		lp1	Satt252_Satt269	SSR	
		lp2	Satt586-gmrvbp	SSR	
Medicago sativa	Aluminium toxicity		UGAc471_UGAc502	RFLP	Sledge et al. (2002)

Table 3 List of some legumes genetically engineered for biotic stress

Legume target	Biotic stress	Gene(s)	Reference(s)
Arachis hypogaea	Tomato spotted wilt virus (TSWV)	Nucleocapsid from TSWV	Magbanua et al. (2000)
	Sclerotinia minor	Oxalate Oxidase from barley	Livingstone et al. (2005)
Cajanus cajan	*Spodoptera litura*	*cry I E-C*	Surekha et al. (2005)
Cicer arietinum	*Helicoverpa armigera*	*cry1Ac*	Sanyal et al. (2005)
	Callosobruchus maculates *Callosobruchus chinensis*	Alpha-amylase inhibitor from bean	Sarmah et al. (2004)
Glycine max	*Sclerotinia sclerotiorum*	Germin (gf-2.8) fom wheat	Donaldson et al. (2001)
	Bean Pod Mottle Virus (BPMV)	Capsid polyprotein from BPMV	Reddy et al. (2001)
	Heterodera glycines	Chitinase from *Manduca sexta*	Ornatowski et al. (2004)
	Helicoverpa zea *Anticarsia gemmatalis* *Pseudoplusia includens*	*cry1Ac*	Walker et al. (2000)
	Heterodera glycines	Promoter of chalcone synthase from Promoter of Phenylalanine Ammonia Lyase	Narayanan et al. (1999)
	Soybean Mosaic Virus (SMV)	Coat protein gene from SMV	Wang et al. (2001)
Medicago sativa	*Phoma medicaginis*	Resveratrol synthase from *A. hypogaea*	Hipskind & Paiva (2000)
	Phoma medicaginis	Isoflavone *O*-Methyltransferase	He & Dixon (2000)
	Phoma medicaginis	Endochitinase (ech42)	Samac et al. (2004)
	Pratylenchus penetrans	Oryzacystatin I and II	Samac & Smigocki (2003)
Medicago truncatula	Alfalfa Mosaic Virus (AMV)	Virus coat protein from AMV	Jayasena et al. (2001)
Phaseolus acutifolius	*Zabrotes subfasciatus*	Arcelins-1, Arcelins-5	Zambre et al. (2005)
Phaseolus vulgaris	Bean Golden Mosaic Virus (BGMV)	*Rep-TrAP-REn*, *BC1* (viral genes)	Aragão et al. (1998)
Pisum sativum	*Bruchus pisorum*	Alpha-amylase inhibitor (alpha-AI-1)	Schröeder et al. (1995) de Sousa-Majer et al. (2004)
	Pea Seed-borne Mosaic Virus (PSbMV)	Replicase (*NIb*) from PSbMV	Jones et al. (1998)
	Alfalfa Mosaic Virus (AMV)	Coat protein from AMV	Timmerman-Vaughan et al. (2001)