

## Pulses: Improving Crop Productivity by Enhancing Abiotic Stress Tolerance

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### **Abstract**

Pulse crops are leguminous plants whose grains are used exclusively for food. In Asia, Africa and many developing countries, pulses constitute a major source of dietary protein and extensive efforts are being undertaken to improve pulse production. However, due to global climate change, abiotic stresses are increasingly impeding crop production. Conventional plant breeding has contributed tremendously in the development of improved crop varieties, but other biotechnological tools are needed to complement breeding efforts to accelerate development of pulse crop varieties tolerant to abiotic stresses such as drought, salinity, high and low temperatures. Genomics resources such as molecular markers have started to expedite marker-assisted breeding and quantitative trait loci (QTL) introgression in chickpea for drought tolerance. Similarly, transcriptomic resources such as expressed-sequence tags, and expression profiling such as microarrays also contribute to further understand abiotic stress tolerance in pulses and for the development of genic markers. In pulse crops, development of *in vitro* regeneration techniques and transgenics has been slow and more resources need to be allocated to expedite their development. *In vitro* regeneration techniques are also useful for embryo rescue of wide hybrids. Transgenics, although controversial, offer a faster means to develop abiotic stress tolerant pulse crops. While enhancement of abiotic stress tolerance in pulse crops implies higher returns in the developed countries, in developing countries it will contribute to food and nutritional security, and sustainable production. It is therefore encouraging that ICARDA, ICRISAT and CGIAR (Generation Challenge Programme) invest extensively into using new technologies for improvement of pulse crops in these regions of low-input farming.

**KEYWORDS:** abiotic stresses, legumes, genomics, marker-assisted selection, transcriptome profiling, transgenics

## **Pulse crops – definition, major and minor pulse crops**

A pulse is a leguminous crop harvested solely for the dry seed, excluding the crops that are mainly grown for oil extraction (e.g. soybean and peanut). Being leguminous crops, pulses play an important role in crop rotation due to their ability to fix nitrogen. The major pulses include common bean or kidney bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), chickpea, bengal gram or garbanzo (*Cicer arietinum*), cowpea (*Vigna unguiculata*), lentil (*Lens culinaris*), pigeonpea or red gram (*Cajanus cajan*), mungbean or green gram (*Vigna radiata*) urdbean or black gram (*Vigna mungo*), lupins (*Lupinus* spp.), faba bean or broad bean (*Vicia faba*), bambara bean or bambara groundnut (*Vigna subterranea*), vetch (*Vicia sativa*), rice bean (*Vigna umbellata*), moth bean (*Vigna acontifolia*), tepary bean (*Phaseolus acutifolius*), adzuki bean (*Vigna angularis*), lima bean (*Phaseolus lunatus*), and runner bean (*Phaseolus coccineus*). The minor pulses include lablab or hyacinth bean (*Lablab purpureus*), jack bean (*Canavalia ensiformis*), sword bean (*Canavalia gladiata*), winged bean (*Psophocarpus teragonolobus*), guar bean (*Cyamopsis tetragonoloba*), velvet bean (*Mucuna pruriens* var. *utilis*) and yam bean (*Pachyrhizus erosus*).

## **Pulse production – Global and different countries from FAOstat**

During 2008, the pulses were grown in 71.8 million ha, producing 61.5 million tons of dry seeds with an average yield of 856 kg per ha [1]. The FAOstat provides individual crop statistics only for few pulses and clubs remaining pulses in groups. The dry bean group of pulses (includes common bean, lima bean, adzuki bean, mungbean, urdbean, scarlet runner bean, rice bean, moth bean and tepary bean) accounts for one-third of the pulse production. In the remaining production, 16.3% is contributed by pea, 13.8% by chickpea, 9.3% by cowpea, 7.0% by faba bean, 6.7% by pigeonpea, 4.6% by lentil, 1.6% by lupins, 1.6% by vetches and 5.7% by minor pulses. During 2008, 170 countries grew pulses, but about 79% of the pulse area was in Asia (48.4%) and Africa (30.5%). Americas, Europe and Oceania accounted for 15.2, 3.7 and 2.1% of the area, respectively. The major pulses producing countries include India (23.2%), Canada (8.1%), China (8.0%), Myanmar (5.8%), Brazil (5.7%), Nigeria (4.8%), USA (3.1%), Russian Federation (3.0), Ethiopia (2.9%) and Australia (2.8%). Most of the pulses are consumed within the producing countries and the international trade is about 16% of the total production. The major pulse importing countries are India (26.3%), Egypt (4.4%), China (4.0%), Pakistan (3.4%), UAE (3.2%), USA (2.8%), UK (2.8%), Italy (2.8%), Turkey (2.8%) and Brazil (2.7%), while the major pulse exporting countries are Canada (32.3%), USA (11.5%), China (10.7%), Myanmar (7.8%), Australia (6.3%) and France (5.1%).

## **Abiotic stresses affecting pulse crops**

The improvement of crop production in the face of acute global climate changes has become a challenging endeavour. Besides the environmental impact, in many regions of the world, crop production has been limited due to socio-economic and political instability. However, advances in genomics research are expected to contribute greatly to alleviate crop production limitations in

many of these regions, where unfortunately, hunger, malnutrition and poverty are widespread. In the western hemisphere, environment and climate change are likely to have the most negative consequences on crop production. Abiotic stress challenges faced by plants include drought, salinity, flooding, metal toxicity (heavy metals), mineral nutrient deficiency, high temperature, low temperature, freezing temperature, UV stress, photoinhibition and anaerobiosis. All too often several of these may affect plants simultaneously, leading breeders to re-think selection strategies for abiotic stress tolerance by considering selection under multiple stresses. While this recognized strategy is likely to be valuable, the complexity of selection for a number stress tolerance traits can be resource-demanding and time-consuming for a breeding programme. Therefore, numerous studies have been undertaken to understand the abiotic stresses challenges and how they affect plant performance.

Among the abiotic stresses affecting pulse crops, drought is probably one of the major concern, especially in the semi-arid tropic (SAT) regions such as Asia and Africa, where it is considered one of the most detrimental stress for pulse crop production [2]. Drought-tolerance and water-use efficiency are intricately related. Drought refers to the insufficient availability of soil moisture, leading to limitation in the supply of water to a growing plant. In arid and semi-arid areas, rainfall patterns tend to be inconsistent, and when combined with high temperatures, moisture for crop growth becomes limiting [3]. Therefore, water use efficiency by crops is a viable strategy to surmount such conditions and breeding and genomics strategies are being developed to maximize this potential [4]. As a result of water deficit and drought, plants are also adversely affected by increase in soil salinity which leads to disruption of plant water status [5]. Salinity and drought combined pose a major problem for normal plant growth in arid and semi-arid regions [6]. While growing salinity tolerant varieties is useful, it is however important to note that tolerance leads to further retention of salinity levels in the soils, affecting subsequently planted crops [7]. Salinity tolerance is conferred by plant's ability to exclude, as opposed to partitioning of ions within the plant, without affecting its performance significantly [8, 9]. Proper management practices are therefore necessary to sustain agricultural production under such adverse soil conditions. Cereals are generally more tolerant to saline conditions than legumes [10] and could therefore be used in crop rotations to minimize build up of salt in soils. In addition, it has also been suggested that evaluation for salinity tolerance be performed at vegetative as well as reproductive stages of growth, since in crops like chickpea salinity affects both these stages and sensitivity is more pronounced at podding stage [11].

Soil fertility can also be limiting for crop production and is due to depletion and degradation of soil nutrients [12]. Therefore, alternative methods for supplementing soils need to be undertaken. Use of fertilizers can be prohibitive for resource-deprived farmers in developing countries and organic supplements are likely to be the best solutions in this situation [13, 14]. When availability of nutrients is low, varieties capable of maximum nutrient use efficiency would be valuable. However, in recent years nutrient uptake with relevance to the

symbiotic arbuscular mycorrhiza (AM) and their associations to abiotic stresses are being more important [15]. There have been extensive studies on the beneficial effects of AM:plant associations for sustainable cropping of temperate crops (for reviews see [16, 17] and research applications for tropical agriculture are being undertaken. For example, mycorrhization has been shown to help with uptake of phosphorus and other nutrients (for reviews see [13, 15]) and also allows plants to tolerate abiotic stresses such as heavy metals [18] and salinity [19]. In *Cajanus cajan*, it has been shown that the AM: root association led to accelerated acquisition of phosphorus by a plant [20]. Similarly, in *Glycine max* [21] and *Cajanus cajan* [22] it has been shown that nutrient uptake and growth is improved under salinity stress due to associations with AM. Radiant frost, is another common abiotic stress experienced under more temperate climatic regimen for cool season pulse crops such as chickpea, lentil, faba bean and field pea [23].

### **Mechanisms underlying stress tolerance – a generalized picture**

Plants, being sessile in nature, need to perceive and adjust as needed to abiotic stress challenges. However, plants can only withstand a certain level of stress and once the optimum threshold is surpassed, cellular and metabolic functions become perturbed leading to sub-optimal performance. For crops, this sub-optimal performance essentially leads to reduced yield. Plants have nonetheless adapted to or have been selected for adaptation to abiotic stresses over time, but further tolerance is required to meet the ever-increasing abiotic stress challenges. Abiotic stresses include those adversities perceived by plants when exposed to drought, salinity, cold, heat, anaerobiosis, heavy metals, light intensity/UV and nutrient limitations and essentially limit crop productivity. These abiotic stresses in essence disturb the homeostatic equilibrium within the plant (for review see [24]). Prior to the availability of genomics tools, a one-gene approach was used to attempt to explain abiotic stress response in a “cause and effect” strategy. However, tolerance to abiotic stresses is complex and in spite of the identification of numerous genes with potential roles in abiotic stress responses, further understanding and dissection of the cascades of events that lead to the ability to withstand such stresses still need to be understood. Genome-wide expression profiling approaches have enabled the elucidation of the roles of many of the genes induced in response to abiotic stresses (for review see [25]). Interestingly, in leguminous crops genome-wide studies for abiotic stress tolerance are very few and done on model legumes such as alfalfa and soybean (e.g., [26-28]). Such studies are important to identify and establish network(s) involved in stress response pathways, which could eventually be manipulated to minimize crop losses due to abiotic stresses [29], especially for pulse crops such as lentil, chickpea, pigeon pea and common bean. Nonetheless, there have been some recent genomics studies on some of the major pulse crops for abiotic stress tolerance.

Generalized stress responses in plants begin with sensing of a stress by the primary sensor, followed by a signaling cascade of events, with calcium being the second messenger. The occurrence of a multiplicity of physiological, biochemical and molecular events in response

to abiotic stresses is well known, including accumulation of intracellular compounds such as nucleic acids, proteins, carbohydrates and amino acids. The series of events from perception of stress to signal transduction to phospho-protein cascades and transcription factor activation/suppression leading to induction of stress-responsive genes, and allowing the plant to respond to perceived stress either as a defensive or protective reaction, is also well elucidated [30]. However, the initial perception and perturbation of cellular function may vary, depending on the abiotic stresses, although some of the stress response pathways are common to various types of abiotic stress challenges such as drought, salinity, osmotic stress and cold [31-33]. For example, drought, salinity, and low, high and freezing temperatures lead to membrane integrity disruption, generation of reactive oxygen species (ROS), accumulation of toxic by-products, photosynthetic process dampening and nutrient uptake reduction [34]. At the molecular levels cross-talks and specificities of signalling pathways also exist in response to abiotic stresses (for review see [35]). In *Arabidopsis*, for example, a histidine kinase was induced in response to salt and considered to be a osmosensor [36]. As a generalized response, elevated levels of calcium in response to a number of abiotic stresses as an early signal or second messenger has also been well established (for reviews see [37, 38]). A number of sensors related to calcium-regulated proteins such as calmodulin [39-41], calcium-dependent protein kinases (CDPKs) [42] and calcium-regulated phosphatases [43, 44] have also been associated with signal transduction pathways in response to different abiotic stresses. The CDPKs, however, have been shown to be stress-specific due to occurrence of many isoforms [45]. Similarly, among the  $Ca^{2+}$ /phosphatase interactions, occurrence of phosphatase isoforms point to stress-specific roles [43]. Thus, due to the multiple roles of calcium sensors by way of different isoforms conferring specificity, they are also central to cross-talks among various abiotic stresses [35].

Another important component of the signal transduction pathways in response to biotic and abiotic stresses and plant hormone signalling and cell division involves the mitogen-activated protein kinases (MAPK) cascades [46-49]. The MAPK cascades have been extensively studied and characterized in higher plants for their roles as signalling molecules [50]. In *Arabidopsis* sequenced genome survey 20 MAPKs, 10 MAPK kinases and 60 MAPK kinase kinases were identified [51]. The regulation of activity of proteins by MAPKs can occur in two possible scenarios; in the first scenario, an activated MAPK phosphorylates nuclear transcription factors, cytoskeletal components and/ or other kinases and in the second scenario other regulatory proteins and MAPK components interact physically, irrespective of phosphorylation state [49]. Once the signal transduction pathway has served its purpose and the downstream response has been elicited, inactivation of MAPKs need to occur and phosphatases reset the signalling pathways by dephosphorylating the MAPKs [52]. Furthermore, phosphatases are able to hold the MAPKs in the cytoplasm or nucleus [53], effectively terminating the signal [54].

Downstream to the signal transduction pathways, induction of transcription factors is invoked, some of which integrate another level on complexity in the cross-talks among different

abiotic stresses. A well-elucidated example of this cross-talk is provided by low temperature, drought and abscisic acid induction of genes by these transcription factors [55, 56]. The promoters of some of these cold- or drought-induced genes contain the CRT (C-repeat element) or DRE (drought-responsive element), respectively, and as characterized in *Arabidopsis* [56-58] are targeted by the *DREB1* or *CBF* transcription factors under low temperature exposure and by *DREB2* under salt or drought exposure. The cross-talk among the low temperature, drought and salinity signalling pathways has been the subject of many studies using the *RD29A* gene of *Arabidopsis*, whose promoter contains the C-repeat element [59]. *RD29A* promoter::*GUS* fusion constructs in *Arabidopsis* leaf protoplasts were shown to be induced by low temperature and drought as a result of over-expression of *DREB1* and *DREB2*, respectively, indicating integration of two different signal transduction pathways, leading to the expression of one gene [57]. With the use of global transcriptomic profiling approaches mostly in *Arabidopsis*, it has been clearly demonstrated that under any type of stress up to 25% of the genome is differentially regulated and that irrespective of the stress applied, a group of 59 genes, 21 of which were transcription factors, were induced [60].

### **Strategies to enhance abiotic stress tolerance - Conventional**

The improvement in productivity of pulse crops has had tremendous success over the years due to the availability of genetic resources and breeding knowledge [61]. While the combination of breeding and diverse germplasms has contributed to yield increments, enhancement to abiotic stresses has proven to be more challenging using conventional approaches. This is mainly due to the quantitative nature of inheritance to tolerance against abiotic stresses. Nonetheless, plant breeders have been able to develop varieties tolerant to some extent to abiotic stress challenges by using various strategies from conventional breeding methodologies to germplasm collections to mutagenic approaches.

### **Breeding**

Plant breeding as an expedited form of evolution has contributed tremendously to the development of new improved crop varieties. It has been viewed as an art in many instances due to the breeder's ability to sometime recognize intuitively certain attributes in breeding lines which would make a variety more suitable. Over time, as emphasis was placed on nutrition, adaptation to environments, quality and economic return, selection became primordial in initiating plant breeding programs [62]. It can therefore be stated that early gatherers were in effect the first breeders since they intentionally selected for desirable traits such as palatability of plant products and ease of harvest during the domestication process of plants, without having much of an impact on the general structure of crop plants [63]. Prior to the re-discovery of Mendel's laws of genetics, breeders were deemed to be successful based on their ability to carefully observe and identify variations showing improved qualities for further advancing as varieties [62]. However, despite advances in areas of genomics, plant breeding will continue to

be the essence of all crop improvement strategies. Plant breeding essentially involves identification of genetic variability and combining this variability to generate plant types with desirable attributes. However, due to the erosion of genetic diversity, there has been a need to mine for variability from other sources. Thus from traditional breeding from the use of germplasm resources from wild species, land races and distant relatives, plant breeders have created new variability by chemical and physical mutagenesis. The phenomenon of somaclonal variation recognized in plant cell cultures [64] also became potential sources of variation.

Application of scientific principles to classical breeding approaches occurred with the re-discovery of the laws of inheritance [65]. Increasing adaptation of cultivars to specific environments were sought by assessing increase in productivity through identification of sources of variability in local germplasm resources or through introduced land races or breeding lines from other breeding programs, wild species or genera. If further variability was needed, plant breeders screened for spontaneous mutations or induced mutations physically or chemically. Such variant genotypes subsequently became parental lines in hybridization experiments and used for qualitative or quantitative trait selections. Depending on the traits under selection, the breeder sets realistic objectives to maximize identification of desired traits in progeny of the crosses. The qualitative and quantitative nature of the traits as well as their heritability needs to be taken into consideration. Thus, plant breeders set objectives in order to determine selection criteria, aid in the choice of breeding method and choice of parents and indicate when selection must begin. The objectives of the plant breeder should also reflect the end-user preferences such as the consumer and the producer so that quality-enhanced and marketable varieties are produced. The general scheme of breeding outlined above is no different in pulse crops [61]. Furthermore, breeding programs have drawn from knowledge gained from model legumes such as *Medicago truncatula*, *Lotus japonicus*, *Glycine max* and *Pisum sativum* to breed for quality and well as other agronomic traits and biotic and abiotic stresses in more complex and less well-understood pulse crops such as faba bean [66].

It is estimated that 60% of the worldwide common bean (*Phaseolus vulgaris* L.) production is drought-prone [67]. At the International Center of Tropical Agriculture (CIAT), a breeding program was initiated for the development of drought resistant breeding lines. Drought resistant lines had significantly higher yields than commercial varieties under drought [67]. Similarly in chickpea (*Cicer arietinum* L.), which is usually grown under rainfed conditions, drought accompanied by heat stress is limiting to growth and a study was initiated to select genotypes resistant to drought/heat with particular reference to selection criteria [68]. The study found that seed weight was least affected by the drought/heat stress conditions and had the highest heritability and could possibly be used in early breeding selections [68]. The study also indicated that days to the first flowering and maturity to escape terminal drought and heat stresses should be evaluated before many other phenological traits [68]. Similar to these studies, deeper rooting systems in chickpea have been associated with better coping against drought [69,

70]. Root traits such as rooting depth and root biomass have also been shown as ideal traits to breed for terminal drought stress tolerance in chickpea [71]. In faba bean (*Vicia faba*), deprivation of water led to increased root growth and root density and both were suggested to be adaptive mechanisms to cope with the limited water supply [72].

### **Mining germplasm resources**

Although breeding efforts have generated numerous improved varieties, erosion of genetic diversity as well as limited availability of germplasm resources has led to a need for the identification of new sources of variability in wild species and land races or by *de novo* generation. Therefore, plant breeders started to screen for mutations for incorporation into breeding programs. This practice led to coining of the term “mutation breeding” and has been practiced for almost a century. Naturally occurring mutants have indeed heralded The Green Revolution due to the use of dwarfing genes found in a Japanese wheat cv. Norin-10. High yielding dwarf rice varieties possessing strong straw were derived from spontaneous semi-dwarf mutation phenotype in a rice cv. Dee-geo-woo-gen from Taiwan.

In leguminous crops, breeders have sought for variation in land races, wild species and wild progenitors. However, this has led to a narrow genetic base for improvement of legumes as these belong to the primary gene pool (GP-1) [73]. The use of the secondary gene pool (GP-2), which consists of species that can potentially be crossed with GP-1, has been used for common bean improvement, but has been challenging for lentil improvement [74]. The availability of large collections of pulse crops germplasm resources around the world such as ICRISAT (India), ICARDA (Syria), CIAT (Colombia) and IITA (Nigeria) now offers the possibility of screening for many traits of interest [74] from collections within these resources. While mostly agronomic and quality aspects have been the focus of breeding employing germplasm resources from these centres, mining for abiotic stress tolerance genes has now become an important component. In an attempt to contribute functional markers for allele mining in chickpea germplasm resources, a root expressed sequence tagged (EST) resource was developed and was suggested to be potentially useful for candidate gene identification for abiotic stress tolerance [75]

### **Variation creation - Traditional mutagenesis and TILLING**

A significant addition to the tool belt of the plant breeder in the early part of the last century was inducible mutagenesis. Although naturally occurring mutations were identified as early as the 1900s by de Vries, with their potential for use in breeding [cited in 76], it was only when physical mutagenesis by X-rays in *Drosophila* [77, 78] and in barley [79] were generated that the new field of induced mutagenesis research started and quickly became part of the field of plant breeding. To date, 3124 mutant varieties are listed with the FAO/IAEA Mutant Varieties Database [80]. Almost 70% of these mutants were advanced over generations and released as new varieties, while the remainder were used in crosses with other varieties [81]. The first



variety to be derived from mutagenic X-ray irradiation was the tobacco cultivar, Chlorina, which was commercially released in 1934 [Tollenaar 1934 cited in 76]. About two decades later, a commercial variety of mustard, cv. Primex, was released from X-ray-induced mutation treatments. Of the direct mutant varieties released, radiation was used in about 89% of these and 75% of the overall mutants in the database were in crops [81]. Mutation breeding for grain legumes has also been widely applied, especially in conjunction with the Joint FAO/IAEA Division [82, 83]. The FAO/IAEA Mutant Varieties Database lists 202 mutant varieties of pulse crops [80] and were generated for mostly agronomic improvement, with a few for abiotic stress tolerance such as drought tolerance in *Cajanus cajan*, cold resistance in *Cicer arietinum* and salinity tolerance in *Lens culinaris*.

Induced mutations gained new impetus in the genomics era, with the specific targeting of known genes. The TILLING (Targeting Induced Local Lesions IN Genomes) [84] strategy has become widely applicable for variation mining in crop plants. The TILLING method enables identification of single base pair changes in genes of interest [85]. Seeds are generally mutagenized with ethylmethane sulfonate (EMS). M1 plants grown from these seeds are selfed, planting individual M2 seeds for DNA extraction and cataloguing M3 seeds. PCR amplification for the gene of interest is done on pooled DNA from the M2 plants. PCR products are denatured and upon re-annealing heteroduplexes are formed. Denaturing-HPLC was originally used to analyze heteroduplexes for mutations [84, 86]. Subsequently, treatment of the heteroduplexes with an endonuclease, CELI, which specifically cleaves mismatches between mutated and non-mutated variant heteroduplexes was used [87]. CELI, which was extracted from celery, recognizes single base mismatches and cleaves on the 3'-side of the mismatch [88]. Electrophoretic separation of cleaved heteroduplexes allows identification of mutations. Mutant plant is then identified by screening DNA from individual samples constituting the pool. EMS generally causes G/C to A/T transitions and the randomly distributed G/C to A/T transitions in *Arabidopsis thaliana* account for up to 99.5% of mutations [89]. TILLING populations are available for *Arabidopsis thaliana* [85], *Lotus japonica* [90], maize [91], barley [92], wheat [93, 93-96] and oat [97]. In legume crops, TILLING populations have been slow to be created and has been restricted to the model legume *Lotus japonica* [90] and in soybean [98]. However, in recent years TILLING populations have been produced for pulse crops including common bean [99, 100] and pea [101]. In order to extend our capabilities to mine for abiotic stress tolerance genes in other major pulse crops such as lentil, chickpeas, cowpea and pigeonpea, TILLING populations need to be developed. For example, in the *Medicago* TILLING population, a leucine-rich repeat, *Srlk* mutant gene, was found not to limit root growth in response to salt stress [102]. Therefore, TILLING populations for pulse crops will be very valuable to mine for abiotic stress genes.

### **Strategies to enhance abiotic stress tolerance – Biotechnology and genomics**

Plant breeding approaches have resulted in the successful development of a number of different commercial varieties of crops. Even today, plant breeding is pivotal in the development of improved varieties. However, erosion of naturally occurring genetic variability has limited the development of newer cultivars with improved qualities by plant breeders [103]. The toolbox of plant breeders has therefore expanded with the use of novel germplasm resources, made available from both *de novo* as well as from induced sources. More recently, the availability of TILLING populations in many crops has further generated sources of variability for traits of interest, although still rudimentary for pulse crops. The TILLING approach is likely to expedite breeding programs due to the variability of a number of defined mutants for traits of interest. Breeding for abiotic stress tolerant crops is a major initiative around the world. However, the quantitative nature of inheritance of abiotic stress tolerance renders this task challenging. Genetic mapping strategies are valuable tools for locating genomic regions associated with abiotic stress tolerance and quantitative trait loci (QTL) mapping has become an integral part of breeding efforts for such complex traits. Other technology-driven tools for pulse crop improvement include *in vitro* culture systems and the phenomenon of somaclonal variation and transgene technology. Besides, these the functional analysis of genes involved in abiotic stress tolerance, including EST resources are important for developing breeding strategies to enhance abiotic stress tolerance.

### **Genetic mapping and QTL analysis**

The complex genetic nature of inheritance to abiotic stress tolerance traits such as drought, salinity and low temperature and the difficulty of phenotyping for such traits under field conditions along with high genotype x environment interactions, were initially daunting [2]. However, with the advent of molecular markers and marker-assisted selection (MAS) tools, there has been an enhanced ability to more effectively select for traits for incorporation into new varieties. More recently the ability to identify quantitative trait loci (QTLs) markers for polygenic traits has expanded marker-assisted breeding. The repertoire of molecular markers has grown considerably over the years from the tedious RFLP types to the more versatile RAPD and AFLP type markers. However, nowadays SSR, SNP and DArT markers have become widely accepted for use. In leguminous crop breeding, these varieties of markers have been employed with varying combinations for biotic as well as abiotic stress tolerance selection, more success being achieved for biotic stress MAS as in soybean for resistance against cyst nematode [104] or for resistance against common bacterial blight in pinto bean [105]. Nonetheless, there have been successful reports of MAS for abiotic stress tolerance or towards achieving that goal by way of identification of QTL. For example, selection for drought tolerant common bean genotypes has been shown using five RAPD markers with improved performance under stressed conditions [106, 107]. In cowpea, QTL associated with drought response phenotypes were identified [108, 109]. Similarly, root trait QTL for drought-avoidance were identified in chickpea and will likely contribute to expedite development of varieties for enhanced drought avoidance [71]. Frost

tolerance QTL have been identified for faba bean and suggested to be valuable in future for efficient screening of large populations [110]. Several winter-hardiness QTL have also been identified in lentil, with one QTL being found to be common to all environments tested [111]. In pea, the flowering locus *Hr* was found to colocalize with a major winter-frost tolerance QTL, thereby making it ideal for MAS [112]. Similar to drought MAS, limited information is also available for MAS for salinity tolerance in pulse crops compared to other legumes such as soybean. Salt tolerance QTL identified in wild and cultivated soybean were found to be conserved and accounted for a large dominant effect [113]. SSR marker alleles flanking a major salt tolerance QTL were also identified in soybean that could be used for MAS for salinity tolerance [114], including during seedling growth [115].

One powerful use of MAS has been suggested for gene pyramiding in the development of crop plants with tolerance to multiple stresses [116] or for introgression of multiple QTLs for a specific abiotic stress [117]. While gene pyramiding and QTL introgression have been shown for cereals for both biotic and abiotic stresses, efforts are currently underway for achieving the same in pulse crops, particularly for abiotic stress tolerance. This was mainly due to lack of abiotic stress related QTL in pulse crops. In common bean, resistance to rust and anthracnose was developed by using marker-assisted backcrossing [118]. Introgression of multiple QTL for root morphological characteristics associated with drought tolerance was shown in rice [119] and it is likely that similar approaches will be successful in pulse crops in the near future as suggested from chickpea root trait QTL studies for drought tolerance [71]. It is foreseen that in the next five years with major QTL being identified for abiotic stresses in pulse crops, the relevance of pyramiding and QTL introgression will increase dramatically. In chickpea, a hot spot region that affects several traits (root length density, root biomass, shoot biomass) contributing to drought tolerance has been identified from two mapping populations (ICC 4958 × ICC 1882; ICC 283 × ICC 8261) segregating for root traits (Gaur et al. unpublished). This region contributes up to 36% phenotypic variation for both root and shoot biomass, and root length density. Terminal drought is the major constraint to chickpea productivity, particularly in the semi-arid tropics where it is generally grown rainfed on residual soil moisture after the rainy season. Root traits, particularly rooting depth and root biomass, are known to play an important role in drought avoidance through more efficient extraction of available soil moisture. This genomic region flanked by SSR markers TAA 170 and ICCM 0249 has been introgressed into three cultivars (JG 11, KAK 2 and Chefe) using marker assisted backcrossing (MABC). While TAA 170 and ICCM 0249 markers were used for foreground selection, eight AFLP primer combinations were used for background selection. BC3F4 progenies are available and will be evaluated along with donor and recipient parents in both irrigated and rainfed conditions. Marker-assisted breeding for root traits is expected to improve precision and efficiency of breeding for drought tolerance in chickpea.

### **Transcriptomic resources**

Gene expression profiling approaches have had tremendous impact on obtaining global snapshots of genes under any particular condition of plant growth, be it spatial, temporal, developmental or environmental. While such expression profiling approaches have been routinely conducted for all major crops for abiotic stress challenges, in pulse crops examples are now only emerging. In chickpea, 2800 root-trait and drought-responsive ESTs were developed and annotated [120]. More recently, a set of 20162 drought- and salinity-responsive chickpea ESTs and gene-based markers have been developed [121]. Similarly, in pigeonpea 9888 fusarium wilt- and sterility mosaic disease-responsive ESTs were developed [122] and the pigeonpea genomics initiative is already starting to provide more resources for pigeonpea improvement [123].

Other transcriptomic approaches have included differential display PCR (DDRT-PCR), cDNA-AFLP and microarrays, although the latter has been mostly restricted to model legumes for abiotic stress transcriptome profiling. Using DDRT-PCR drought-modulated gene(s) in common bean were examined and 8.7% of the 1200 DDRT bands were found to be regulated by drought [124]. Similarly, cDNA-AFLP profiling in cowpea during biological nitrogen fixation under heat stress revealed 55 transcripts which were up-regulated and nine which were down-regulated by heat stress [125]. Subsequently, using the transcript-derived fragments as probes against a cowpea heat-stressed root nodule cDNA library, it was shown that two of the full length clones isolated coded for a small heat shock protein gene and a nodulin gene [126]. Studies such as the cDNA-AFLP transcriptome analyses are valuable since they allow for gene discovery under abiotic stress challenges, as recently reported in wheat, wherein temporal and spatial specificity of induced transcripts under low temperature exposure occurred [127]. The cDNA-AFLP profiling is a low-cost alternative for gene discovery, especially in laboratories with limited resources [103] and will be valuable for pulse crop transcript profiling under abiotic stress challenges.

Compared to other crops, microarray-based studies in pulse crops have also been lagging behind. Most microarray studies relating to abiotic stresses have been done in model legumes such as *Medicago truncatula* or soybean. For example, in *Medicago truncatula* a time-course experiment was conducted for salt stress in roots using the Affymetrix *Medicago* GeneChip and MtED (*Medicago truncatula* Expression Database) was suggested to be a useful resource for studying abiotic stress in other legumes [128]. A similar experiment for root apex responses to salt compared to whole roots response to salt using a 16K+ microarray chip showed that there was a 30-fold expression difference in transcription factor genes, suggesting different spatial adaptive response within the roots to soil environments [26]. Microarray analysis of aluminium stressed root tips of *Medicago truncatula* revealed novel genes involved in resistance or tolerance to Al resistance [129]. Such studies need to be extended to pulse crops, especially since EST resources are becoming increasingly available.

Next-generation sequencing (NGS) is the most recent technological addition to expediting genome sequencing. Genomic sequencing information is valuable for different purposes such as gene identification and molecular marker development in varieties of interest. With the availability of a reference genome as in *Medicago* [130], single nucleotide polymorphism (SNP) markers can be developed for other varieties. Even if a reference genome is not available, NGS can be performed. For example in chickpea, using Solexa tags of root tissues of drought tolerant and drought sensitive genotypes, 5.2 and 3.6 million reads, respectively were generated, with the identification of about 500 SNPs [131]. Chickpea transcriptome has also been sequenced with short reads on Illumina Genome Analyzer platform and will be valuable for marker development and gene identification [132].

The role of small RNAs in post-transcriptional regulation of gene expression is now well established, including their roles in abiotic stress tolerance (for reviews see [133-135]). A few encouraging reports have recently shown the roles of miRNAs in abiotic stress tolerance in pulse crops. For example, cowpea miRNAs have been identified and their potential roles in salinity stress tolerance due to differential expression in roots have been shown [136]. Stress-responsive miRNAs were also identified in common bean subjected to nutrient deficiency stress and manganese toxicity [137]. Another study has identified and validated miRNAs in different organs of common bean and under growth conditions such as drought, abscisic acid treatment and *Rhizobium* infection [138]. Recently, eight potential miRNAs from horsegram (*Macrotyloma uniflorum*) were identified by computational mining of EST database at the NCBI and shown to be novel plant miRNAs involved in a variety of responses including environmental stress [139].

### **Transgenic approaches**

Transgenic approaches, as controversial as they may be, offer perhaps one of our fastest means for the development of abiotic stress tolerant pulse crops. However, the transgenic technology can also complement functional genomics studies to validate expression of cloned genes related to abiotic stress tolerance. In pulse crops, both *in vitro* culture and genetic transformation were slow to be developed. This was mostly because of heightened interest in cereals such as rice and wheat, because of their important contributions to the energy supply of human beings. Although cereals were generally considered recalcitrant to *in vitro* culture and genetic transformation, successes were achieved due to tremendous resources which were allocated for the production of transgenic cereals. Similar resources and efforts need to be directed towards pulse crop transformation to increase the efficiency and reproducibility of transformation systems for pulse crops.

Since the first report of *Agrobacterium*-mediated delivery of genes to produce transgenic plants and Mendelian transmission of the transgene in 1983 [140], a number of other gene delivery methods have been reported. Although *Agrobacterium*- and particle gun-mediated

delivery are the most popular methods, other methods have also been successfully used to produce transgenic plants for commercial applications and/or basic studies to understand plant growth and development. The availability and versatility of different DNA delivery methods are becoming important for pulse crop improvement, since transcriptomic resources are becoming increasingly available. Furthermore, although sequences of known functions in the databases can be used for homology-based prediction of gene function of unknown sequences, more precise functions of the genes of interest are often difficult to ascertain, except by a transgenic approach [141]. Therefore, the availability of high-throughput gene transfer systems for economically important pulse crops has become highly necessary for rapidly assessing gene function. Such transformation systems are routine in model systems such as *Arabidopsis*, but are still lacking for many economically important crop plants. This is particularly critical for pulse crops which have been relatively recalcitrant to transformation. Of particular potential for pulse crop transformation, is the *in planta* transformation system. Such non-tissue culture transformation approaches by *Agrobacterium* have been reported in some leguminous crops and are promising for pulse crops transformation. For example, pricked peanut embryo axes were transformed by inoculating them in *Agrobacterium* suspension [142]. Seedlings or flowering plants of *Medicago truncatula* were also transformed by vacuum infiltration of *Agrobacterium* [143]. The electroporation-mediated transformation of nodal axillary buds of pea, cowpea and lentil and production of transformed plants is encouraging for further refinement of this strategy for transforming pulse crops [144].

### ***In vitro* regeneration and transformation**

Notwithstanding the recalcitrance of pulse crops to tissue culture and transformation, there are many successful reports (for review see [145]). *In vitro* culture as such, prior to being a target for use in transformation, was used for embryo rescue in wide hybridizations and for *in vitro* selection. For example, inter-specific hybrids of lentil were rescued by embryo culture [146, 147]. Similarly, hybrid plants from a cross between *Phaseolus vulgaris* L. and *P. lunatus* L. were obtained by embryo rescue and confirmed by rDNA analysis [148]. In chickpea, although limited success for inter-specific hybridization and embryo rescue were initially encountered [149], some success has been obtained [150]. Embryo rescue was also used to produce hybrids between *Cajanus platycarpus* × *C. cajan* [151, 152]. Considering that tolerance to abiotic stresses exists in many wild species of pulse crops, hybridization between cultivated species and their wild counterparts, followed by embryo rescue is likely to be a strategy worth exploring. Several wild species of *Cicer* have been shown to be tolerant to cold or drought [149, 153, 154]. One such species, *C. pinnatifidum*, tolerant to cold and belonging to the tertiary gene pool, was crossed with *C. arietinum* and the hybrid was rescued by embryo culture [150]. Successful hybrids have also been produced by embryo rescue in cultivated lentil × wild lentil sp. [155, 156]. Hybridization among several *Vigna* sp. and mungbean cultivars, followed by embryo

rescue gave successful hybrids with potential for use in improving these *Vigna* sp. for abiotic stress tolerance [157].

*In vitro* selection for tolerance to biotic and abiotic stresses was also an area of extensive research in the seventies and eighties. Combined with the phenomenon of somaclonal variation [64], *in vitro* selection was deemed to contribute new variation to breeding programmes. The idea that plant cells can be treated as micro-organisms and subjected to selection pressure existed for a long time and essentially breeders could perform selection on cultured cells and tissues under appropriate selective agent to identify and regenerate plants from tolerant or resistant cell lines. While this approach was widely explored for disease-resistance selection by employing fungal toxins or crude culture filtrates from fungal cultures shown to contain fungal toxins in tissue culture medium, advances for abiotic stress tolerance selection have also been attempted [158]. Abiotic stress was generally applied as NaCl or mixture of salts for salt tolerance selection and as polyethylene glycol (PEG) or mannitol for drought tolerance selection. For example, salt tolerant *Vigna radiata* plants were regenerated by selective NaCl pressure [159, 160]. As with transgenic studies, *in vitro* selection studies in pulse crops have equally lagged behind and need to be revisited. Studies on *in vitro* selection for salt tolerance in other leguminous crops such as soybean [161] and alfalfa [162] have been done. Some studies in *Vigna* have used callus for selection to drought by employing PEG in the culture medium [163], but no drought tolerant regenerated plants have been reported to date.

As with other biotechnological approaches for pulse crop improvement, genetic transformation strategies have likewise been slow to be implemented. ICRISAT has taken a leading role in recent years at improving mandated pulse crops for abiotic stress tolerance, especially drought tolerance and include use of genetic transformation technology [164]. Transgenic chickpea lines over-expressing a mutagenized *pyrroline-5-carboxylate synthetase* (*P5CS*) gene led to elevated proline levels under water deficit in the greenhouse, but no significant effect on yield was observed, although transpiration efficiency was modestly improved [165]. A similar strategy in soybean, but using the *L-Δ<sup>1</sup>-pyrroline-5-carboxylate reductase* (*P5CR*) gene showed elevated accumulation of proline under stress and the better ability to metabolize proline after re-watering [166]. Interestingly, a *P5CS* gene from *Vigna aconitifolia*, altered by site-directed mutagenesis to prevent feed-back inhibition of proline [167], was used to produce transgenic tobacco plants with increased drought tolerance [168]. Such studies are encouraging and especially since the *P5CS* gene was cloned from a pulse crop. Other studies have targeted transcription factors regulating expression of many genes upon abiotic stress challenges. The most extensively studied transcription factors are the *dehydration-responsive element-binding/C-repeat-binding* (*DREB/CBF*) from *Arabidopsis* and their involvement in multiple abiotic stresses [57, 169]. Transgenic peanut plants expressing *DREB1A* from *Arabidopsis* showed increased transpiration efficiency under limiting water availability

[170]. Production of transgenic pulse crops tolerant to abiotic stresses is thus possible, but resources need to be allocated to realize this possibility.

### **Concluding remarks**

Pulse crop improvement for tolerance to abiotic stresses still needs tremendous resources to be invested to cater to world food security and nutrition. While in the developed countries pulse crop improvement for abiotic stresses will lead to profitable returns on production, in developing countries it will improve food security, nutrition and sustainable production. Furthermore, due to low-input farming in many developing countries and the occurrence of drought and low soil fertility, the need to develop improved pulse crop varieties is urgent. The Generation Challenge Programme (GCP, <http://www.generationcp.org>) under the umbrella of the Consultative Group on International Agricultural Research (CGIAR) is precisely aiming to provide molecular biology-based resources for crop improvement in developing countries. Some of the pulse crops targeted for development of molecular markers for stress tolerance include cowpeas, beans and chickpeas for the sub-Saharan African and South Asian regions. Such programmes and those undertaken by ICARDA and ICRISAT are likely to provide much required impetus to pulse crop improvement for abiotic stress tolerance. However, there is a need for more international partnership, especially from developed countries already extensively researching pulse crop improvement. The accessibility of next-generation sequencing is opening up genomics resources hitherto not completely utilized and such resources will be valuable to expedite pulse crop improvement. In the short term, transgene technology needs to be explored and adopted to deliver transgenic pulse crops with abiotic stress tolerance.

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