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Grain Legumes: Biotechnological Interventions in Crop Improvement for Adverse Environments

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Abstract

Grain legumes are the major food crops grown and consumed mostly by the poor farmers as an important source of protein in the drylands of the world. The ensuing climate change has posed serious potential threats to the cultivation of these crops that are important for the sustainable livelihoods of the poorest of the poor in these regions. There is evidence to suggest that the ecological dynamics and equilibriums are likely to be affected as a result of changing climate, either by making these crops susceptible to new diseases or by increasing the intensities of diseases, pests, and parasites. Despite many uncertainties, there is a growing consensus that these adversities could lead to an overall increase in the disease and pest pressure besides harsher abiotic stresses. Since most of the grain legumes have a narrow genetic base and levels of resistance to some biotic and abiotic constraints are low, making crop improvement an overarching research-for-development challenge for maximizing the benefits that grain legumes offer to smallholder farmers. Running against the headwinds, grain legume research has been immensely benefited by applications of modern biotechnological tools and approaches that have the potential to develop solutions for destructive diseases, besides making headway against the complex problems of drought. Similarly, identifying novel genes/traits and assessing their suitability as candidate genes for genetic engineering options will be important for future breeding programs in order to achieve remarkable impacts in these grain legume crops globally. This chapter mainly provides a comprehensive picture of the different biotechnological interventions adopted for addressing various constraints in gain legume productivity and improvement, highlighting the pitfalls and possible solutions that can be taken through an integrated approach to combat the altered environmental conditions.

16.1
Introduction

With the advent of twenty-first century, agriculture will not only be forced to compete for land and water with sprawling urban settlements but will also be required to serve on other major fronts: adapting and contributing to the mitigation of climate change, the most pronounced adverse condition on the doorstep. Climate change will affect the four dimensions of food security: availability, accessibility, utilization, and stability [1]. According to the estimation of a FAOs discussion paper, by 2050, developing countries may experience a decline of between 9% and 21% in overall potential agricultural productivity as a result of global warming [1]. Alarmingly, the poorest regions will be exposed to the highest degree of instability of food production due to substantial decline in agricultural productivity, including labor productivity, leading to increases in poverty and mortality rates.

Food and agriculture sectors will be significantly impacted by adverse climatic features that are likely to include increased occurrence of extreme heat (temperature and duration), short-term fluctuations, seasonal oscillations, sudden discontinuities, and long-term variations [2]. Despite many uncertainties and unknowns, there is a growing consensus that ecological dynamics and equilibriums are likely to be affected and there will be an overall increase in the abundance and diversity of invertebrate pests—and pest pressure—as habitats become more favorable for their establishment and development and new niches appear [1]. Furthermore, studies suggest that hosts and pathogens may be brought together in new locations and contexts, bringing new threats to crops, livestock, and aquaculture systems and new challenges, with the accompanying need for significant human and financial investments to address the challenges.

The strongest negative impact of these adversities on agriculture is expected in sub-Saharan Africa [1]. It has been estimated that climate change may reduce African potential agricultural output up to the 2080–2100 period by between 15 and 30% [1]. This denotes that SAT areas are likely to be in most vulnerable condition, the poorest and most food insecure region is also expected to suffer the largest contraction of agricultural incomes resulting in an increased dependence on food imports.

16.2
Grain Legumes: A Brief Introduction

Grain legumes or pulses are a widely adapted group of crop plants and occupy an important place in the world food and nutrition economy. The present world production of grain legumes is estimated to be 50 million metric tons and, at an average price of $400 per metric ton, its total value would amount to $2 billion [1]. They are important constituents in the diets of a very large number of people, especially in the developing countries, and are good sources of protein that help to supplement cereal diets, improving their protein nutritive value [3]. 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 [4]. Although most legumes are consumed as dry grains, immature green pods or green seeds are also used as vegetables providing substantial quantities of minerals and vitamins to the diet. Although many species and subspecies of legumes are known, only about a dozen of them are important as commercial food crops. Beans and peas each account for about 25% of the total production of legume crops. Chickpea and broad beans rank next in importance. Some of the legumes, however, are of only regional or local importance.

Field pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), and grasspea (*Lathyrus sativus* L.) are collectively known as the cool season food legumes. These groups of legume crop plants grow vegetatively during the cool season and flower and produce seeds as day lengths become progressively longer. Carbonized remains indicate that peas, lentils, and chickpeas were domesticated in the Near East arc and were cultivated with the cereals as early as the seventh millennium BC [5]. From the presumed center of origin, peas spread to the cool-temperate areas of Central and Northern Europe and from there were introduced into the Western Hemisphere soon after Columbus [6, 7].

The major grain legumes such as soybean, peanut, pigeonpea, common bean, and cowpea are warm season legumes better adapted to the humid regions. The warm season legumes are characterized by epigeal germination, a period of rapid vegetative growth, followed by flowering when day lengths become progressively shorter during the growing season. In contrast, the cool season pulses have hypogeal germination, a period of rapid vegetative growth, followed by flowering when day length becomes progressively longer.

16.3
Major Constraints for Grain Legume Production

The adversities in growing conditions of the grain legumes pose threat and affect the adaptability and productivity that reportedly has shown an increased severity depending on the type and the specific crop location. Furthermore, crops under abiotic stress are usually more susceptible to weeds, insects, and diseases, which considerably increase the losses [8].

16.3.1
Biotic Stresses

The major biotic stresses affecting legumes include viruses, fungal, and bacterial diseases, insect pests, nematodes, and parasitic weeds that drastically decrease the grain legume production. Considerable progress has been made toward the successful management of important diseases of most legume crops through the search for host resistance. Though sources of resistance to many important diseases have been found and are being used to breed agronomically acceptable

cultivars with good levels of resistance, pathogens are highly variable, making breeding for resistance to be a long-term objective. Hence, combined disease resistance is required in most legume production systems. This has proved relatively easy to attain in some cases, but rather difficult in other cases.

16.3.1.1 Fungal Diseases

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 [9]. Several rust species can infect grain and forage legumes, most of them belonging to the genus *Uromyces*, such as *Uromyces appendiculatus* on common bean, *Uromyces ciceris-arietini* on chickpea, *Uromyces pisi* on pea, *Uromyces striatus* on alfalfa, *Uromyces viciae-fabae* on faba bean, lentil, and common vetch, and *Uromyces vignae* on cowpea. Also, rust species belonging to other genera can be major problems on legumes such as *Phakopsora pachyrhizi* and *Phakopsora meibomiae* on soybean or *Puccinia arachidis* on groundnut [10]. Asian rust (*P. pachyrhizi*) is a severe disease that causes important yield losses in soybean and is spreading rapidly around the world [11–13]. Lack of natural sources of resistance makes this disease a good candidate to be solved using biotechnological tools. Normally, legume rust epidemics begin late in the season, when pod filling starts, so yield components are only slightly affected by the infection and losses are usually low. However, when the infection starts early in the season, severe epidemics can occur [14]. Similarly, powdery mildew caused by *Erysiphe pisi* is an important fungal disease in several legumes and has a worldwide distribution being particularly important in climates with warm, dry days and cool nights, adversely affecting yield and quality [9]. Severe infection may cause 25–50% yield losses. In contrast, 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 [9].

The major necrotrophic fungal disease of various grain legumes is Ascochyta blight, caused by *Ascochyta rabiei*, reportedly the most important fungi affecting pea and chickpea [15, 16]. Similarly, Botrytis gray mold caused by *Botrytis cinerea* is of lesser importance, but is also a widespread foliar disease problem in grain legumes. Besides these, there are several soil-borne diseases that are common among legume crops [17]. Most of these attack the seedling stage of the crop and are referred to as damping-off diseases, several examples include damping-off, generally caused by either *Rhizoctonia solani* or *Pythium* spp., which can result in up to 80% of plant death [18, 19] and Fusarium root-rot (caused by *Fusarium* spp.) causing severe seedling losses especially in common bean and lentils [20, 21]. In most growing areas of the world, Fusarium wilt (FW) (caused by *Fusarium oxysporum*) is a major constraint, affecting seedlings and adult plants where it causes leaf chlorosis, wilting, and death in chickpea [16, 22] and lentil in particular. Other important

soil-borne diseases such as southern stem rot (*Sclerotium rolfsii*) and the white mold (*Sclerotinia sclerotiorum*) cause both seedling and pod rots in warmer and cool weather, respectively [23].

16.3.1.2 Viral Diseases

During the last two decades, viruses have emerged as devastating pathogens, particularly in the tropics and subtropics, causing huge economic losses and threatening crop production to grain legumes. 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. 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%. Similarly, the peanut stem necrosis disease epidemic that resulted in the death of young groundnut (*Arachis hypogaea* L.) plants occurred in the rainy season of the year 2000 in Anantapur district, Andhra Pradesh, India, where the crops were usually grown on 0.7 million ha. The disease affected nearly 225 000 ha and the crop losses were estimated to exceed Rs. 3 billion (US$65 million). Similarly, sterility mosaic disease (SMD) is the most damaging disease of pigeonpea in the Indian subcontinent, Bangladesh, Nepal, Thailand, Myanmar, Sri Lanka, and China with annual losses of over US$ 300 million. These epidemics caused by re-emerging and newly emerging viruses are becoming frequent even in regions that were earlier free from these viruses. The major contributory factors for the emergence and spread of new virus diseases are the evolution of variants of the viruses and the increase in the vector population. For example, genomic recombination in gemini viruses, not only between the variants of the same virus but also between species and even between genera, has resulted in rapid diversification.

16.3.1.3 Insect Pests

Insects are another important biotic stress faced by many legume crops. They cause important damages through both by direct feeding as vectors and by providing infection sites for pathogens. 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 [24], and weevils such as *Apion godmani* and *Zabrodes subfasciatus* in warm season legumes [25, 26].

16.3.1.4 Parasitic Weeds

A number of parasitic plants have become weeds, posing severe constraints to major crops including grain legumes [27]. One such example is of *Orobanche foetida*, which is widely distributed in natural habitats in the Western Mediterranean area parasitizing wild herbaceous leguminous plants, also considered an important agricultural parasite in the faba bean in Beja region of Tunisia. *Orobanche minor* is of economic importance on clover that is grown for seed and has recently become a problem on red clover in Oregon [10, 28]. Similarly, *Striga gesnerioides* and *Alectra*

vogelii cause considerable yield reduction of grain legume crops, particularly cowpea, throughout semiarid areas of sub-Saharan Africa [29].

16.3.2
Abiotic Stresses: A Threat to Grain Legumes

Abiotic stress broadly include multiple stresses such as heat, chilling, excessive light, drought, water logging, wounding, ozone exposure, UV-B irradiation, osmotic shock, and salinity. Some of these stresses like drought, extreme temperature, and high salinity dramatically limit crop productivity. Drought stress is particularly important in context of the grain legumes, such as peanut (*A. hypogaea*), Brazil nuts (*Bertholletia excelsa*), and faba bean (*V. faba*). Moreover, drought–*Aspergillus* interaction results in occurrence of preharvest aflatoxin contamination in these crops [30–32]. Likewise, waterlogging due to a combination of unfavorable weather conditions and suboptimal soil and irrigation techniques can result in severe yield losses in grain legumes [33, 34]. Several of the abiotic stresses associated with legume crops can also directly affect symbiotic interactions and therefore limit legume growth [35].

Drought problems for legumes are likely to worsen with the projected rapid expansion of water-stressed areas of the world from 28 to 30 countries today to 50 countries encompassing 3 billion people by 2030 [36]. Thus, there is a crucial need to increase drought tolerance in legumes; however, increasing salinity tolerance is a parallel requirement in many areas. The more drought-tolerant legumes, such as cowpea, are deeply rooted and the reduced leaf size with thickened cuticles might be responsible for the reduced water loss under stress.

16.3.2.1 Heat Stress
Heat stress due to increased temperature is an agricultural problem in many areas in the world. Transitory or constantly high temperatures cause an array of morphoanatomical, physiological, and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. The adverse effects of heat stress can be mitigated by developing crop plants with improved thermotolerance using various genetic approaches.

16.3.2.2 Salinity
Soil salinity is one of the main abiotic stresses that plants encounter more frequently. It is expected that by 2050 more than 50% of all arable lands will be saline [37]. Soil salinity affects total nitrogen uptake and soil nitrogen contribution [38], leading to reduced yield besides limiting a constraint to their symbiotic functioning [39]. In addition, inhibition of rhizobial growth has been recognized at NaCl levels that were toxic, but not lethal to the host plants, strains visibly possessing variations in their sensitivity to salinity [39]. Nevertheless, the progress in the direct screening for grain yield under saline conditions has been hampered by the low heritability, polygenic control, epistasis, significant genotype $\times$ environment (G $\times$ E) interaction, and quantitative trait loci (QTIs) $\times$ environment (QTL $\times$ E) interaction [40].

16.4
Biotechnological Interventions in Grain Legume Improvement

Breeding for resistance or traditional breeding program has served for a long time as the only solution to the problem regarding the adverse environmental condition. However, lack of knowledge regarding genetic variation and in-depth study on molecular mechanism behind stress tolerance largely affected the progress of the crop improvement programs. Recent advancements on different biotechnological approaches opened a new era of stress biology and widened the scenario of traditional breeding programs using molecular breeding approaches. Pure line breeding, population breeding, mutation breeding, and wide hybridization have been used for development of new varieties of legume crops and have led to incremental improvements in the yield potential of these crops.

Most of the grain legumes have a narrow genetic base [41], and levels of resistance to some biotic and abiotic constraints are low. Since the overarching research-for-development challenge in grain legumes is to apply crop improvement to maximize the benefits that grain legumes offer to smallholder farmers, running against the headwinds grain legume researchers have nonetheless achieved remarkable impacts globally by increasing yields and developing solutions for destructive diseases besides making headway against the complex problems of drought. Applications of the developed genomic tools and modern breeding approaches potentially will shorten the breeding cycles and eventually lead to development of superior cultivars. Similarly, identifying novel genes/traits and assessing their suitability as candidate genes for genetic engineering options will be important for future grain legume breeding programs.

Keeping in view the adversities faced by the grain legumes, this chapter deals with the novel and efficient modern breeding methods/biotechnological tools for accelerating the grain legume improvement programs. Various biotechnological approaches for crop improvement of the major grain legumes such as groundnut, chickpea, pigeonpea, soybean, common bean, cowpea, and lentil are being discussed in detail in the following sections.

16.4.1
Groundnut

Groundnut (*A. hypogaea*) (also known as peanut, earthnut, and monkey nut or goobers) is an important oilseed cash crop containing 36–54% oil, 16–36% protein, and 10–20% carbohydrates, cultivated in over 100 tropical and subtropical countries of the world [1]. About 70% of the world's peanut is produced in the SAT and India has the largest peanut growing area with 6.7 million ha (27.3%) and stands second in the production at 6.5 million tons (18.2%). Since 80% of the crop is grown under rainfed conditions by resource-poor farmers, the rainfall pattern during the presowing months and the availability of substitute high-value oilseed crops like soybean and sunflower of short durations requiring less water have had a significant negative impact on acreage allocation decisions of the farmers. Moreover, a

big gap exists between the realized yield and potential yield of peanut at both subsistence and commercial systems of production in Asia and Africa due to both abiotic and biotic factors. The major abiotic factors affecting peanut production include drought, high temperature, low soil fertility, low soil pH, and iron chlorosis. Among the biotic factors, fungal diseases, virus diseases, bacterial wilt disease, aflatoxin contamination, nematodes, foliar insect pests, and soil insect pests are important [42].

Groundnut crop improvement by conventional breeding has been struggling to meet the demands of increasing population, especially in seed quality improvement and developing virus- and insect-resistant varieties. Several advanced research institutes or groups are working to apply modern biotechnology for groundnut improvement in developing countries. These include marker-assisted selection (MAS), tissue culture, embryo rescue, and genetic modification techniques.

16.4.1.1 Biotechnology for Tolerance to Abiotic Stresses

Drought is the major cause for low and erratic pod yield in peanut that contributes to over 6.7 million tons loss in annual world peanut production [43], resulting in estimated monetary losses of over US\$ 520 million annually [44]. Yield losses in peanut due to water deficits vary depending on timing, intensity, and duration of the deficit, coupled with other location-specific environmental stress factors such as high irradiance and temperature [45]. Due to the scarcity of available water in SAT regions, drought management strategies, whether agronomic or genetic, therefore, need to focus on maximizing extraction of available soil moisture and the efficiency of its use in crop establishment: growth, biomass, and grain yield [46].

The first genetic map for cultivated groundnut (*A. hypogaea*), an amphidiploid (4X) species was developed at ICRISAT that demonstrated its utility in molecular mapping of QTLs controlling drought tolerance-related traits as well as in establishing relationships with diploid AA genome of groundnut and model legume genome species [47, 124].

In order to develop a genetic linkage map for tetraploid cultivated groundnut, a total of 1145 microsatellite or simple sequence repeat (SSR) markers available in public domain as well as unpublished markers from several sources were screened on two genotypes, TAG 24 and ICGV 86031 that are parents of a recombinant inbred line mapping population. As a result, 144 (12.6%) polymorphic markers were identified that amplified 150 loci. A total of 135 SSR loci could be mapped into 22 linkage groups [47, 124]. More recently, nearly 700 genes were identified in subtractive cDNA library from gradual process of drought stress adaptation in groundnut [48]. A high-density oligonucleotide microarray for groundnut has also been developed using 49 205 publicly available ESTs and tested the utility of this array for expression profiling in a variety of groundnut tissues [49].

Recently, a straightforward laboratory protocol used acquired thermotolerance (ATT) in groundnut seedlings as a measure of one mechanism of heat stress tolerance. Sixteen genotypes were evaluated for acquired themotolerance in two independent experiments. A change in the temperature sensitivity of chlorophyll

accumulation was used as an indicator of acquired thermotolerance [50]. Interestingly, another study indicated that lipoxygenase and 1L-myo-inositol-1-phosphate synthase, which aid in inter- and intracellular stress signaling, were more abundant in tolerant genotypes of groundnut under water deficit stress. Here, the acetyl-CoA carboxylase, a key enzyme of lipid biosynthesis, increased in relative abundance along with a corresponding increase in epicuticular wax content in the tolerant genotypes suggesting an additional mechanism for water conservation and stress tolerance. In addition, there was a marked decrease in the abundance of several photosynthetic proteins in the tolerant genotypes along with a concomitant decrease in net photosynthesis in response to water deficit stress [51].

Drought is a very complex trait, involving the concerted action of many genes and gene families, a feature that renders engineering drought tolerance very challenging. There have been very few efforts on developing groundnut transgenics for abiotic stresses. Transgenic groundnut plants transformed with AtNHX1 gene were reported to be more resistant to high concentration of salt and water deprivation than their wild-type counterparts. Salt and proline level in the leaves of the transgenic plants were also much higher than that in the wild-type plants [52]. At ICRISAT, groundnut was transformed using a single regulatory gene (DREB1A transcription factor), which in turn regulates the expression of downstream genes leading to the activation of many functional genes [53, 54]. Preliminary results of these transgenics showed that several events acquired the capacity to extract more water from the soil profile or had altered leaf conductance. O the 50 independent transgenic events thus produced, 6 transgenic events have single transgene insert and variable transpiration efficiencies (TE) and desirable root traits were selected for further evaluation of pod yield under both drought and fully irrigated conditions, in a series of greenhouse and field environments. These trials were also the basis of a comprehensive study of the component traits leading to drought adaptation. The transgenic peanuts were evaluated based on their effectiveness in (i) capturing the water; (ii) effective usage of captured water for producing biomass via photosynthesis; and (iii) converting assimilate into a harvestable form based on the equation $Y = T^{*}TE^{*}HI$. Nevertheless, outputs of these study have the potential to draw us closer to a transgenic solution to this global problem by reconciling both molecular and agronomic approaches toward a common focus of groundnut breeding for drought tolerance.

16.4.1.2 Biotechnology for Resistance to Biotic Stresses

The major biotic stresses for groundnut include the foliar fungal diseases, leaf spot (early and late), and rusts. Besides, seed and soil-borne diseases like collar rot, stem rot, and dry root rot have also been identified as important in groundnut. Among viral diseases, bud necrosis (BND), stem necrosis (PSND), peanut mottle (PMV), and peanut clump (PCV) are considered to be of economic importance in groundnut. With regard to the insect pests, a wide range of pests like leaf miner, tobacco caterpillar, white grub, jassids, thrips, aphids, red hairy caterpillar, and termite are known to cause serious damage to groundnut crop [55].

A considerable number of SSR sequences for resistance to various diseases have been identified from peanut genome by several research groups [56–65] The SSR markers have been identified and characterized for association with resistance traits such as rust and late leaf spot resistance [66, 67] and resistance to *Ralstonia solanacearum* [68] and *Sclerotinia minor* [69]. Genetic linkage maps with SSR markers have been constructed for diploid AA genome [59], BB genome [70], tetraploid AABB genome derived from a cross of cultivated with amphidiploids [71], and tetraploid AABB genome in the cultivated peanut [72].

For virus resistance, the most common gene, which has been targeted at early stages of virus multiplication, is the coat protein gene of the target virus. The first ever-transgenic groundnuts, resistant to the peanut clump virus (IPCV) wide spread in West Africa and pockets of Asia, have been developed [73]. Besides, exploitation of pathogen-derived resistance (PDR) to groundnut rosette disease (GRD) by using GRAV-*cp* gene to induce host plant resistance to GRD has been carried out at ICRISAT and is undergoing testing in Africa. Transgenic plants of groundnut varieties, Gajah and NC 7, have been developed using one of the two forms of PStV coat protein (*cp*) gene that exhibited high levels of resistance to peanut stripe potyvirus (PStV), wide spread in East and Southeast Asia [74].

Similarly, transgenic options to combat peanut bud necrosis disease (PBND) and PSND include development of transgenic groundnut plants expressing either the nuleocapsid gene or the coat protein gene in groundnut. Biotechnological interventions to address the problem of spotted wilt of groundnut caused by tomato spotted wilt virus (TSWV) have been underway by various groups. Milla *et al.* [75] used AFLP markers to establish marker trait association for TSWV resistance in groundnut. These technologies are in different stages of development and have a potential to be incorporated in peanut breeding programs for improvement following their regulatory approvals. Since protection of transgenic plants against many of these viruses is under both RNA- and protein-mediated control [76], efforts on harnessing antisense and RNAi technology for resistance to these viruses are being pursued by various groups [77, 78].

Besides viruses, fungal diseases in groundnut are the most significant limiting factor causing more than 50% yield losses throughout the world. Early leaf spot caused by *Cercospora arachidicola* S. Hori (*Mycosphaerella arachidis* Deighton), late leaf spot caused by *Phaeoisariopsis personata* Berk. & M.A. Curtis (*Mycosphaerella berkeleyi*), rust (*P. arachidis*), crown rot (*Aspergillus niger* Teigh.), collar rot caused by *Aspergillus* spp., root rot caused by *Macrophomina phaseolina*, stem rot caused by *S. rolfsii*, and yellow mold (*Aspergillus flavus* and *Aspergillus parasiticus*) causing aflatoxin contamination are the major fungal diseases affecting groundnut crop. Aflatoxin contamination in top groundnut-producing states of the United States caused average annual losses of US$ 26 million to its southeastern groundnut industry [79]. Chemical control and conventional breeding have yielded only limited success. Although wild relatives of groundnut possess resistance to foliar diseases to the level of even immunity [80–82], the interspecific hybridization has not been highly successful in introgression of the desirable traits where desired due to complexity of inheritance and several inherent breeding barriers [83, 84]. This

narrow genetic basis of the cultivated groundnut *A. hypogaea* L. hampers the development of improved varieties through conventional breeding.

To overcome these bottlenecks, transgenic options for groundnut crop improvement for various diseases have been carried out by various groups. Transgenic groundnut expressing a tobacco chitinase gene II [85] and rice chitinase and an alfalfa glucanase gene [86] have been shown to possess enhanced resistance to the late leaf spot and *Sclerotinia* blight, respectively. Glucanase gene from tobacco introduced into groundnut (PR protein from heterologous source) showed enhanced disease resistance to *C. arachidicola* and *A. flavus* [87]. Transgenic plants of groundnut carrying mustard defensin gene showed increased disease resistance to *C. arachidicola* Hori. and *P. personata* [88]. Similarly, overexpression of barley oxalate oxidase gene in transgenic groundnut showed enhanced resistance to oxalic acid producing fungi, *S. minor* [89].

Since aflatoxin contamination of groundnut not only causes significant economic hardship for producers but also poses a serious health threat to humans. Transgenic interventions for enhancing host plant resistance to *A. flavus* infection have been carried out in groundnut. There have been various studies carried out for developing transgenic groundnut plants either overexpressing and/or downregulating different genes for resistance to *A. flavus* and aflatoxin [90–92].

So far, groundnut is virtually unexplored at the genomic level because of the large genome size (2800 Mb/1C) and complication and hence expressed sequenced tags (EST) have been considered to be a quick and economical approach to identify important groundnut genes involved in defense response against fungal infections, also providing data on gene expression and regulation [93, 94]. Efforts have been made to identify and characterize the peanut EST regulated during interaction with the fungus *Cercosporidium personatum* (causing late leaf spot) using suppressive subtractive hybridization (SSH) to prepare the subtracted cDNA libraries [95]. Utilizing the genomic and proteomic tools, genes and proteins associated with *A. parasiticus* and drought stress were identified in groundnut [96–98]. Such genes have a potential to be used for enhanced fungal disease resistance in groundnut through marker-assisted selection in breeding or by direct upregulation or downregulation of the target gene using genetic engineering.

16.4.2
Chickpea

Chickpea is the most important food legume of semiarid tropics (SAT) and taxonomically one of the closest crops to the model legume *Medicago*. Chickpea, from their region of domestication in the Near East, quickly spread to the Indian subcontinent where it became a principal pulse crop and a dietary mainstay [99]. Chickpea was successfully introduced to Central and South America and to the western United States [99]. Chickpea is the major pulse crop in the Indian subcontinent where it is produced on nearly 7 million ha [99]. Chickpea's yield potential is limited by a series of biotic and abiotic stresses, including Ascochyta blight, Fusarium wilt, drought, cold, and salinity. It is mostly grown under

rainfed conditions in arid and semiarid areas around the world. Despite growing demand and high-yield potential, chickpea yield is unstable and productivity is stagnant at unacceptably low levels. Together with low temperatures and water stress, high salinity is responsible for crop losses of millions of tons of various legume (and other) crops and continuously deteriorating environmental conditions combined with salinity stress to further compromise chickpea yields. Hence, major yield increases in chickpea could be achieved by development and use of cultivars that resist/tolerate abiotic and biotic stresses. To accelerate molecular breeding efforts for the discovery and introgression of stress tolerance genes into cultivated chickpea, functional genomics approaches and/or transgenics are rapidly growing. Recently, a series of genetic tools for chickpea have become available that have allowed high-powered functional genomics studies to proceed, including a dense genetic map, large insert genome libraries, expressed sequence tag libraries, microarrays, serial analysis of gene expression, and transgenics and reverse genetics.

16.4.2.1 Biotechnology for Tolerance to Abiotic Stresses

Chickpea (*C. arietinum* L.), a deep-rooted legume grown in semiarid regions of the world, is liable to terminal drought, which impacts plant growth and descends global annual yield stability. Chickpea varieties susceptible/tolerant to abiotic stress have been characterized under abiotic stress conditions (especially drought stress), although very little is known about the genes involved in abiotic stress responses. Nevertheless, the characterization of genes involved in the differential behavior of these cultivars may constitute a good basis to extrapolate these results to other grain legumes. In order to identify and understand the molecular mechanisms of tolerance to abiotic stress such as drought, a total of 10 996 ESTs were generated from 10 cDNA libraries derived from root tissues of drought-tolerant (ICC 4958) and drought-sensitive (ICC 1882) chickpea genotypes grown under control and drought stress conditions. The drought-responsive ESTs along with 7439 salinity-responsive ESTs generated from salt stress challenged tissues of JG 11 and ICCV 2 genotypes of chickpea, and 7097 public domain chickpea ESTs were comprehended to derive 9569 unigenes (2431 contigs and 7138 singletons) and were used to identify and design EST-based SSR and SNP markers. The availability of such large number of ESTs derived from different resources provided an added advantage for computational SNP discovery, first by assembling ESTs derived from different individuals and then scanning the multiple sequence alignments (MSA) of the contig to find sequence differences that correlate with the source of the EST, providing opportunities to understand the genetic mechanisms underlying stress responses in this legume. (http://www.icrisat.org/bt-gene-discovery.htm).

Similarly, a transcriptional profiling study in chickpea was carried out using cDNA microarray approach under drought, cold, and high salinity to look at the gene expression in the leaf, root, and/or flower tissues in tolerant and susceptible genotypes [100]. Besides, superSAGE analysis for gene expression in chickpea roots in response to drought resulted in sequencing of a total of 80 238 of 26 bp tags [101] (Among these tags, a total of 7532 (43%) UniTags were more than 2.7-fold

differentially expressed, and 880 (5.0%) were regulated more than 8-fold upon stress resulting in unambiguous annotation of 22% (3858) of these tags. This is the first study to prove the potential of SuperSAGE technology for molecular breeding in the nonmodel crops. Interestingly, in this year the same group has done an excellent modification of this technique as deepSuperSAGE, and raised a huge amount of data against abiotic stress [101].

In sharp contrast to the importance of chickpeas as staple food and industrial raw material, the salt responses at the transcriptome and proteome levels had been dealt with at only very low throughput until some years ago, that is, tens or at the most hundreds of genes had been considered [100]. Molina *et al.* [102] applied deepSuperSAGE to detect early global transcriptome changes under salt-stressed chickpea. The salt stress responses of 86 919 transcripts representing 17 918 unique 26 bp deepSuperSAGE tags (UniTags) from roots of the salt-tolerant variety INRAT-93 2 h after treatment with 25 mM NaCl were characterized. Of the total 144 200 analyzed 26 bp tags in roots and nodules together, 21 401 unique transcripts were identified. Of these, only 363 and 106 specific transcripts were commonly upregulated or downregulated (>3.0-fold), respectively, under salt stress in both organs, witnessing a differential organ-specific response to stress. These results demonstrated that ROS-scavenging and ROS-generating pathways undergo strong global transcriptome changes in chickpea roots and nodules 2 h after the onset of moderate salt stress (25 mM NaCl). These newly identified transcript isoforms are potential targets for breeding novel cultivars with high salinity tolerance [102].

Most of the earlier understanding of dehydration-responsive cellular adaptation in chickpea has evolved from transcriptome analyses and the comparative analysis of dehydration-responsive proteins, particularly proteins in the subcellular fraction, is limiting. Bhushan *et al.* [103] have initiated a proteomics approach to identify dehydration-responsive extracellular matrix (ECM) proteins in JG-62, a drought-tolerant variety of chickpea where the dehydration-responsive temporal changes of ECM proteins revealed 186 proteins with variance at a 95% significance level. This study, for the first time, demonstrated that over a hundred ECM proteins are presumably involved in a variety of cellular functions, namely, cell wall modification, signal transduction, metabolism, and cell defense and rescue, and impinge on the molecular mechanism of dehydration tolerance in plants. Another study provided insights into the complex metabolic network operating in the nucleus during dehydration in chickpea [104]. Approximately 205 protein spots were found to be differentially regulated under dehydration. Mass spectrometry analysis allowed the identification of 147 differentially expressed proteins, presumably involved in a variety of functions including gene transcription and replication, molecular chaperones, cell signaling, and chromatin remodeling. The dehydration-responsive nuclear proteome of chickpea revealed a coordinated response, which involves both the regulatory and the functional proteins [104].

Efforts on enhancing the drought tolerance using transgenic interventions have also been carried out in chickpea, where transgenics carrying *P5CSF129A* gene encoding 1-*pyrroline-5-carboxylate synthetase* were constitutively expressed

for overproduction of an osmolyte proline, which is known to have a role in osmotic adjustment and cell protection under water deficits [105]. In an another effort, *DREB1A* cDNA from *Arabidopsis thaliana*, capable of transactivating DRE-dependent transcription in plant cells under the control of stress-inducible *rd29* promoter, was introduced to a popular chickpea cultivar for improving drought and salinity tolerance in this important pulse crop (K.K. Sharma, unpublished results).

16.4.2.2 Biotechnology for Resistance to Biotic Stresses

In chickpea, genetics of resistance to Ascochyta blight [106–109] and Fusarium wilt [110–114] have been extensively analyzed. A comprehensive overview of previous genetic mapping efforts in chickpea is available [115]. The advent of sequence-tagged microsatellite sites (STMS) markers [116, 117], however, provided the opportunity to integrate the different available maps.

Cool and wet weather conditions are typical for Mediterranean winters, favoring the development of Ascochyta blight that is caused by the necrotrophic fungus *A. rabiei* (Pass.) that affects all aerial parts of chickpea. Although sources of resistance have been identified [106], the development of stable blight-resistant lines would allow a shift to sowing into the rainy season. The genetics of resistance to Ascochyta blight has been extensively analyzed because the disease is of great agronomic and economic importance. Nevertheless, more durable resistance could probably be achieved by pyramiding of resistance genes via MAS and is currently a major challenge for chickpea breeders [118].

Among the many insect pests, the legume pod borer, *H. armigera*, is the most devastating pest damaging chickpea in Asia, Africa, and Australia. Despite the efforts made over the past four decades to breed crops for resistance to insects, the progress has been less than satisfactory in many cases. The resistance to *Helicoverpa* in chickpea has so far been found to be low to moderate. Therefore, it is imperative to evaluate the use of biotechnology to provide alternative and sustainable levels of resistance to this insect pest. Genetic transformation has been an important strategy to introduce the insecticidal protein genes from the bacterium *Bacillus thuringiensis* (*Bt*) and other heterologous sources for improving the resistance of chickpea. Although extensive work has been carried out in developing transgenic plants with *Bt* and other insecticidal genes to combat the insect pest *H. armigera*, there has not been a major breakthrough in controlling this devastating pest in this important pulse [119]. Since there has not been a major breakthrough in identifying events showing a much higher expression of the insecticidal protein sufficient to cause a 90–95% insect mortality under natural conditions, efforts are also focused on using several promoter–gene combinations for efficient expression of the *Bt* genes. Efforts are being made to use SARs (scaffold attachment regions) and MARs (matrix attachment regions) for high gene expression and exploring possibilities of using suppressor genes such as *AC2* to enhance the *Bt* gene expression in this crop. There is a need to produce larger numbers of transgenic events of chickpea using various *Cry1* and *Cry2* gene constructs to obtain one or more agronomically acceptable insect-resistant chickpea lines.

Aphis craccivora, the homopteran group of sucking pest, causes major damage to chickpea by extracting nutrients from the phloem [120]. Apart from the direct damage, they transmit different viruses that cause damaging diseases to the crop. Expression of ASAL driven by a phloem-specific *rolC* promoter was carried out in chickpea transgenics followed by their evaluation by binding analyses of its respective *cis*-elements with host nuclear transcription factors. The novel in planta bioassay conducted on *A. craccivora* revealed that insect survival and fecundity decreased significantly in T1 plants in comparison to the untransformed control chickpea [120]. Apart from pod borer and aphids, bruchids cause substantial loss during storage in chickpea [121]. The cowpea weevil (*Callosobruchus maculatus*) and azuki bean weevil (*Callosobruchus chinensis*) infest chickpea seeds heavily. To address this, α-amylase inhibitor gene isolated from *Phaseolus vulgaris* was introduced into chickpea cultivar K850 through *Agrobacterium*-mediated transformation. Results of bioassays revealed a significant reduction in the survival rate of bruchid weevil *C. maculatus* reared on these transgenic chickpea seeds [122].

While transcriptomics studies including microarrays have been used extensively for transcriptional profiling of plant responses to biotic and abiotic stresses, most of these are focused on either biotic or abiotic stresses, making it difficult to construe the genes that may be common to both biotic and abiotic stress responses. Such information may help molecular breeders to develop cultivars with broad-spectrum resistances to these stresses. A 768-featured boutique microarray was employed to compare the genes expressed by chickpea in response to drought, cold, and high salinity and the fungal pathogen *A. rabiei* and 46, 54, 266, and 51 differentially expressed transcripts were identified, respectively [123]. The expression of common genes indicated crosstalk in the genetic pathways involved in responses to these stress conditions. The response of ICC 3996 to *A. rabiei* was more similar to that of high-salinity stress than to drought or cold stress conditions [123].

16.4.3
Pigeonpea

Pigeonpea (*Cajanus cajan*), an important food legume crop in the semiarid regions of the world and the second most important pulse crop in India, has an average crop productivity of 780 kg/ha that is relatively lower than many other legumes. The low yields may be attributed to nonavailability of improved cultivars, poor crop husbandry, and exposure to a number of biotic and abiotic stresses in pigeonpea growing regions. Narrow genetic diversity in cultivated germplasm has further hampered the effective utilization of conventional breeding as well as development and utilization of genomic tools, resulting in pigeonpea being often referred to as an "orphan crop legume."

Conventional breeding efforts in pigeonpea crop improvement have been successful in producing improved seed quality and reduction of crop maturity duration. Nevertheless, genetic improvement of pigeonpea has been restricted due to the nonavailability of better genetic resources and strong sexual barriers between

the cultivated and wild species. The recent developments in plant biotechnology have provided immense potential in overcoming some of these constraints, thereby offering opportunities for its successful integration with conventional crop improvement strategies. The breakthrough in pigeonpea genome sequencing [124, 125] has resulted in identification of newer genes related to biotic and abiotic stresses in this legume crop, which can effectively be utilized to find stress-responsive genes in other legume crops through comparative studies. Pigeonpea genome analysis predicted 48 680 genes and also showed the potential role that certain gene families, for example, drought tolerance-related genes, have played throughout the domestication of pigeonpea and the evolution of its ancestors [124].

16.4.3.1 Biotechnology for Tolerance to Abiotic Stresses

Drought, cold, heat, and salinity are the abiotic stresses that affect the pigeonpea yield. Besides, waterlogging, heavy rains, and frost are very harmful for the crop. Hence, improvement of pigeonpea for tolerance to these abiotic stresses is very important for obtaining increases in the harvest index and ultimately the yield. Pigeonpea has remarkable drought tolerance traits, which have been used for the isolation of stress-responsive genes. A recent study identified 75 ESTs obtained from the cDNA libraries of drought-stressed plants, 20 ESTs proved to be unique to the pigeonpea [126]. Expression profiles of selected genes revealed increased levels of m-RNA transcripts in pigeonpea plants subjected to different abiotic stresses. Transgenic *Arabidopsis* lines, expressing C. cajan hybrid proline-rich protein (CcHyPRP), *C. cajan* cyclophilin (CcCYP) and *C. cajan* cold and drought regulatory (CcCDR) genes, exhibited marked tolerance, increased plant biomass, and enhanced photosynthetic rates under PEG/NaCl/cold/heat stress conditions [126]. These genes, as such, hold promise for engineering crop plants bestowed with tolerance to major abiotic stresses [126]. A recent study identified 5692 unique candidate single feature polymorphisms (SFPs) that are microarray-based molecular markers detected by hybridization of DNA or cDNA to oligonucleotide probes, extending the marker repertoire with functional marker systems in pigeonpea [127] With the pigeonpea genome sequence now available, genes need to be confirmed experimentally, but are, nonetheless, candidates that can be used to gain insight into the genetic architecture of pigeonpea's drought tolerance and for screens to identify superior haplotypes for improvement. This will facilitate the assembly and alignment of the sequence of other legume crops as well as the unique genes found in pigeonpea may be exploited to improve other legumes for various traits. These candidate genes are useful resource for undertaking the gene expression analysis as well as development of functional markers for both basic and applied research, especially for drought tolerance in pigeonpea improvement [127].

Although relatively tolerant to drought, pigeonpea is sensitive to photoperiod and temperature. While low temperatures affect the short-duration varieties of pigeonpea, high temperature and photoperiod affect the yield of medium and long-duration varieties rendering them to terminal drought. Likewise, in cool areas, maturity in long-duration pigeonpea is accelerated and severe competition occurs between intercropped maize whose maturity is delayed and pigeonpeas resulting in yield

reduction of both crops. It is proven that membrane lipids hold the key for improvement of photosynthesis under low-temperature and high-temperature stress conditions [128]. Not much progress has been made using transgenic solutions for various abiotic stresses in pigeonpea, which need to be improved for getting high yield varieties for this important grain legume. Moreover, pigeonpea is classified as moderately sensitive to salinity [129], development of salt-tolerant variety could be thus useful for Indian farmers as it is grown predominantly in the states where more than 51% of the saline soils in India are located [130].

16.4.3.2 Biotechnology for Resistance to Biotic Stresses

Modern genomic tools such as molecular markers and candidate genes associated with resistance to biotic stresses such as Fusarium wilt, sterility mosaic disease, and pod borer (*H. armigera*) offer the possibility of facilitating pigeonpea breeding for improving biotic stress resistance. Availability of limited genomic resources, however, is a serious bottleneck to undertake molecular breeding in pigeonpea to develop superior genotypes with enhanced resistance to above-mentioned biotic stresses. FW- and SMD-responsive ESTs in pigeonpea have been analyzed and this information can be used for the development of novel SSR and SNP markers in pigeonpea [131]. For enhancing the genomic resources of pigeonpea against biotic constraints, ICRISAT has developed large-scale SSR markers from BAC (bacterial artificial chromosome)-end sequences (BESs) and their subsequent use for genetic mapping and hybridity testing in pigeonpea [132]. Besides, genomic studies on pigeon pea have been conducted with emphasis on genetic mapping and evaluation of polymorphism using RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), microsatellites, and simple sequence repeats (SSR) for *H. armigera* resistance genes [133].

Bulk segregant analysis (BSA) has been employed to identify simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) markers associated with sterility mosaic disease that is considered to be an important disease of pigeonpea causing substantial loss in yield [134]. From 13 polymorphic AFLP primer combinations between the parents, 2 AFLP primer pairs generated 4 markers. O these, two were reported to be linked in coupling phase to the susceptible dominant allele amplifying only in susceptible individuals that can be effectively used for marker-assisted selection [134]. With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, there have been efforts on using a rice chitinase gene for transformation in pigeonpea [135].

Since conventional breeding methods have not been very successful in producing pest-resistant genotypes of pigeonpea due to the limited genetic variation in cultivated germplasm, ICRISAT has developed an efficient method to produce transgenic plants of pigeonpea through *Agrobacterium tumefaciens*-mediated genetic transformation [136]. Lawrence and Koundal [137] transformed pigeonpea with a cowpea protease inhibitor gene for developing transgenic pigeon pea resistant to chewing insects, mainly pod borers. Similarly, Surekha *et al.* [138] successfully transformed pigeon pea with synthetic *cry* I E-C gene. Transgenic pigeon pea events carrying the *Bacillus thuringiensis cry1Ab* and soybean trypsin inhibitor

(SBTI) genes are being developed and evaluated for resistance to *H. armigera* under greenhouse and field conditions in Patancheru, Andhra Pradesh, India.

16.4.4
Soybean

Soybean is the most valuable legume crop, with numerous nutritional and industrial uses due to its unique chemical composition. With its high protein (40%) and moderately high oil (20%) contents, soybean is the world's main source of vegetable protein and oil, accounting for 55% of all oilseeds produced. The enormous agronomic importance of soybean, coupled with the development of modern molecular biology, has led to an increasing level of activity to develop soybean genomics. The efforts in the soybean research community have led to substantial progress in the areas of molecular marker development, expressed sequenced tag databases, BAC end sequences, microarrays, and efficient genetic engineering capabilities. A physical map for cv. Forrest was completed with NSF support (and a physical map of cv. Williams 82, developed with USB funding, is nearing completion (unpublished)). Thus, soybean is also positioned as a key model for translational genomics in grain legumes.

16.4.4.1 Biotechnology for Tolerance to Abiotic Stresses

Depending on hybrid characteristics, soybeans use about 450–700 mm of water during the growing season [139] with drought reducing its yield by about 40% [140]. The most critical period for water stress in soybean has been reported to be during the flowering stage and the period following flowering. However, despite the large resources committed to soybean breeding, progress in improving drought resistance has been slow for a number of reasons [141]. A large number of QTLs have been identified in soybean for traits related to agronomic, physiological, seed composition, and both biotic and abiotic stress parameters (www.soybase.ncgr.org). However, to date only a few QTLs associated with drought resistance traits have been identified.

In the last decade, considerable progress has been made in developing genomic resources for soybean, including the sequencing of the entire soybean genome of approximately 975 Mb (http://www.phytozome.net/soybean#C). Various types of physical maps have also been using RFLP, RAPD, SSR, and AFLP markers [142]. A high-density genetic linkage map of soybean using EST-derived microsatellite markers was generated using a hybrid between the Japanese cultivar "Misuzu-daizu" and the Chinese line "Moshidou Gong 503" by Japanese researchers [143, 144]. Also, the possibility of segmental duplications in the previously suggested regions of the soybean genome was confirmed by inspecting the colinearity between the genomes of soybean and *Lotus japonicus* [144]. Detailed genetic and physical maps of the soybean genome, which together cover the soybean genome by more than 35-fold, have also been created using microsatellite markers from BAC libraries [145]. The Williams 82 *Bsty*I library containing 92 160 clones with an average insert size of 150 kb covering approximately 12 genome

equivalents has been constructed. A genetic map was then integrated to this physical map by anchoring approximately 1000 SSR and STS markers, developed from expressed sequence tags, which are associated with drought responses, disease resistance, seed development, and composition traits [146]. In addition, a six-dimensional pool array has been recently developed from 49 152 *Bst*γI soybean clones ($\sim$6$\times$ genome equivalents), comprising 208 BAC pools [146]. The integrated genetic and physical map will be useful for comparative genetic analysis, map-based cloning of QTLs of desired traits, and genomic sequencing. Recently, the entire soybean genomic sequence has been released with 66 153 protein-coding loci (http://www.phytozome.net/soybean#C). Besides, a spotted cDNA microarray is available containing 36 000 elements constructed from soybean cDNAs, which were derived from a variety of EST libraries representing a wide source of tissues and organs, developmental stages and stress, and pathogen-infected plants (Vodkin *et al.*, 2004). The ESTs isolated from the subtracted library of drought-stressed soybean root tips and submitted to GenBank will greatly contribute to the stress-specific unigenes for further functional genomics work aimed at better understanding of the drought stress response of the soybean root system [147]. So far QTLs in soybean under water deficit conditions have been reported only for WUE and leaf ash [148]. More studies are needed to identify QTLs that influence root architecture and shoot turgor maintenance. Mapping for new QTL(s)/gene(s) and determination of gene action under drought will likely provide key resources to improve tolerance to drought stress in soybean. There exists a substantial genetic variation in soybean for salt response and QTLs associated with salt tolerance in soybean have been identified [149]. With the availability of mutant populations and major achievements in marker-assisted selection and soybean transformation, it is now possible to study and characterize the genes related to drought resistance, leading ultimately to better soybean productivity.

Besides these efforts on functional genomics, there have been enormous efforts on transgenic interventions for abiotic stress tolerance in soybean. Transgenic soybean plants overexpressing the *Arabidopsis* Δ1-pyrroline-5-carboxylate synthase gene, *P5CS*, showed greater tolerance to drought stress due to an increased free proline level and relative water content and a reduced level of reactive oxygen species, particularly hydrogen peroxide [150–152]. Since this report, much effort has been directed toward isolating drought stress-related genes in all physiological and biochemical aspects of drought stress, TFs, and their respective promoters, which will in turn provide novel tools and resources for the development of engineered soybean with improved drought resistance. The identified soybean candidate genes are usually tested for their ability to enhance drought tolerance in *Arabidopsis* before pursuing their engineering into soybean. Chen *et al.* [153] isolated an *AtDREB* homologous gene *GmDREB2* from soybean, constitutive expression of which increased the survival rate of transgenic plants without growth retardation under water deficit and salinity stress conditions. In addition, a few basic leucine zipper genes encoding bZIP TFs were cloned from soybean and found to be induced by drought and high salt treatments. By overexpressing some of these genes in *Arabidopsis*, the researchers found that transgenic plants have increased

freezing and salt tolerance, but no significant difference in drought tolerance [154, 155]. In two other recent studies, it was found that overexpression of a GmERF TF family member, the *GmERF089* gene, and a chilling inducible *GMCHI* gene promoted enhanced tolerance to drought stress in tobacco and *Arabidopsis*, respectively [156, 157]. Recently, 31 *GmNAC* genes, including the six genes previously identified by Meng *et al.* [158], were identified and cloned from soybean. Systematic expression analysis of these 31 *GmNAC* genes demonstrated that nine genes are dehydration inducible [159]. These nine *GmNAC* genes and their respective promoters are promising tools for genetic engineering to improve drought resistance of soybean, as the NAC family was reported to be a major group of TFs that play a role in root development and stress tolerance in plants [160–163].

Recent reports on transgenic soybean for abiotic stress tolerance include transformation with coding sequence for cyanamide hydratase (Cah), an enzyme that converts toxic cyanamide to urea, from the soil fungus *Myrothecium* [164]. Another report on the constitutive expression of *nectarin1* (*NTR1*) gene from *Brassica campestris* in transgenic soybean resulted in enhanced accumulation of methyl jasmonate (MeJA). *NTR1* gene encodes jasmonic acid carboxyl methyl transferase, which is involved in plant development as it regulates the expression of plant defense genes in response to various stresses such as wounding, drought, and pathogens. The higher levels of MeJA in the transgenic soybean plants conferred tolerance to dehydration during seed germination and seedling growth as reflected by the percentage of the fresh weight of seedlings.

These recent advances in soybean research, ranging from breeding programs to genome sequencing and genomics technologies, provide unprecedented opportunities to understand global patterns of gene expression and their association with the development of specific phenotypes, as well as promising tools for the genetic improvement of closely related species grown in adverse environments by molecular breeding or transgenic approaches. Profiling soybean transcriptome, proteome and metabolites will lay the foundation for a systems biology approach to understand key processes such as growth characteristics, stress responses, and yield. Identification of several root-related and stress-specific candidates could help understand the biochemical networks involved in stress responses. Characterization of these candidates, engineering of selected genes through translational genomics pipeline, and field testing of the transgenics are in the pipelines in different research laboratories.

16.4.4.2 **Biotechnology for Resistance to Biotic Stresses**
As soybean crop acreage increased over the years, the crop "matured" and is now host to numerous pathogens and pests. Soybean cyst nematode and *Phytophthora sojae* are the primary causes of yield loss in most of the soybean production regions followed by charcoal rot, virus diseases, sudden death syndrome, seedling diseases and a mix of leaf blights, stem rots, and other nematodes. In addition to these soybean diseases, eight major insect pests also contribute to yield losses due to feeding and damage to seed quality. This damage is more consistent in the southern United States and sporadic in the Midwest. A new insect threat has emerged with the

newly introduced soybean aphid in the Midwest, where insecticide applications are now becoming a routine practice. Asian soybean rust, a disease that causes serious losses in many parts of the world, was first detected in the continental United States in November 2004 and is caused by the fungus *P. pachyrhizi.* Long known to occur in Asia, the fungus spread to Zimbabwe, South Africa, Paraguay, Brazil, Columbia, and now the United States during the past 10 years. Yield losses in other parts of the world due to soybean rust are reported to range from 10% to 90%.

Soybean has several R-gene and QTL clusters to biotic pathogens already identified and mapped in the soybean genome. It is highly likely, based on other host pathogen systems, which QTLs for the slow rusting (partial resistance) phenotype for soybean rust may map to these regions as well. There have been numerous efforts in the past 10 years to identify new genes and sources of resistance to many of soybean's biotic pests. Biotechnological approaches for the development of genetically engineered soybean lines, which express insecticidal molecules, are being widely studied. Genetic transformation of soybean to induce resistance to lepidopterans using the insertion of Bt toxins dates back to early 1990s [165, 166], where these transgenic soybeans showed resistance toward *Helicoverpa zea*, soybean looper Pseudoplusia, tobacco burworm (*Heliothis virescens*), and velvet bean caterpillar (*Anticarsia gemmatalis*). Moreover, the occurrence of proteinase inhibitors (PIs) as defense-related proteins in soybean have also been reported to inhibit the growth of insect pests larvae, including the coleopteran *Tribolium confusum* (Haq) [167, 168] and other insect species, such as *Anagasta kuehniella*, *Hypera postica*, and *Anthonomus grandi* [169, 170]. Recently, soybean biotechnology has been extended to expressing double-strand RNAs (dsRNA) in order to drive gene silencing in nematodes. The posttranscriptional gene silencing using dsRNA RNAi constructs decreased cyst nematodes in transformed soybean roots [171–173].

The glyphosate-tolerant GM soybean alone corresponds to 52% of all biotech crops planted in world area. Indeed, considering soybean, herbicide tolerance has still been the major aimed trait, with around 10 novel varieties showing tolerance to different chemical compounds in their final steps of R & D pipeline to commercial events. However, there is an obvious need and seed market demand for insect pests and plant pathogens resistance traits in soybean. In a very near future, the first GM soybean resistant to insect pests and nematode will be available as single traits or together with herbicide tolerance (stacked traits). Hence, it is expected that in near future, the production of soybean will be possible with less or none agrotoxic residues or mycotoxins enhancing the soybean quality and crop production.

16.4.5
Cowpea

Cowpea, *Vigna unguiculata* L. Walp., is one of the most important food and forage legumes in the semiarid tropics as well as a valuable and dependable commodity for farmers and grain traders. Of the ∼21 million acres grown worldwide, 80% of cowpea production takes place in the dry savannah of tropical West and Central Africa, mostly by poor subsistence farmers in developing countries. Apart from

improvement of agronomical traits, the biotechnological interventions in cowpea breeding and improvement programs aim toward combating abiotic stress (drought, salinity, and heat) tolerance, photoperiod sensitivity, plant growth type, and seed quality with resistances to the numerous bacterial, fungal, and viral diseases and insect, invertebrate (nematode), and herbivorous pests. However, cowpea being adapted to different environmental conditions could potentially be used as an alternative crop for salt-affected soils [174].

16.4.5.1 Biotechnology for Tolerance to Abiotic Stresses

Cowpea is one of the important food legumes cultivated by poor farmers in Sub-Saharan Africa and Asia known to have a better tolerance to drought and high temperature compared to other legumes [175]. Preserving membrane integrity by avoiding membrane proteins degradation is essential for plants to survive in drought stress and hence gives cowpea an edge over other legumes [176]. Transcriptomic studies suggest that several coping mechanisms exist in cowpea for preventing lipid and proteins degradation and for generating reaction oxygen species (ROS) (superoxide radicals, O_2-; hydrogen peroxide, H_2O_2; and hydroxyl radicals, OH). Several reports indicate that drought-tolerant cowpea cultivars adopt these strategies by maintaining the level of expression of certain genes such as cystatin and aspartic protease and by promoting membrane integrity [176]. The transcripts coding these proteins were isolated in drought-tolerant cowpea cultivars subjected to water deficit and their expression localized in different organs [177, 178]. Interestingly, while phospholipase D is a major lipid-degrading enzyme in cowpea cultivars sensitive to drought, the expression of the gene encoding phospholipase D1 (VuPLD1) moderately increased in the drought-tolerant cowpea cultivars [179]. Moreover, the cowpea-tolerant cultivars have been reported to overexpress the gene that encodes ascorbate peroxidase in the chloroplast, while this enzyme is activated in the cytoplasm, peroxisome, and chloroplast [180]. The isolation and characterization of nine drought-inducible genes (CPRD) from 4-week-old cowpea plants by differential screening have been carried out to elucidate the molecular response of cowpea plants to drought stress [181]. In another study, two novel cDNAs (one of them VuNCED1 gene encodes the 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme involved in ABA biosynthesis) have been isolated by a series of differential screening under drought stress. Moreover, several transcripts known as CPRD (cowpea clones responsive to dehydration), CPRD8, CPRD14, CPRD22, and VuNCED1 encode a 9-*cis*-epoxycarotenoid dioxygenase responsible for ABA (abscisic acid) biosynthesis which is highly expressed in cowpea during drought, high salinity, and heat stresses [181, 182]. To understand the molecular bases of thermotolerance, differentially expressed transcripts from cowpea nodules have been identified following subjection to heat shock treatment [183]. These transcripts showed homologies with low molecular weight heat shock proteins, wound-induced proteins, disease-resistant protein, xylan endohydrolase isoenzyme, and different housekeeping genes.

Although much has been studied about the drought tolerance mechanisms in cowpea, a very little perusal of literature is available regarding the biotechnological

approaches exclusively meant for salinity tolerance in cowpea. Often the genes identified as thermotolerant or drought resistant are found to be involved with salinity stress. Salt stress affects cowpea (*V. unguiculata* L. Walp) varieties at different growing stages and according to a recent study in Cuba, *Vigna* genotypes show significant differences in their tolerance to salinity [184].

The recent influx of molecular markers has enhanced our understanding of cowpea's genome structure and organization. Studies based on RAPD, DAF, and SSR markers revealed a low genetic diversity among cowpea varieties and molecular polymorphism between drought tolerance and sensitive varieties, and also between the higher and lower nitrogen fixing cowpea accessions [185–188]. The first attempt to build a genetic map of cowpea was performed by Fatokun *et al.* [189] by using a population resulting from a cross between an improved genotype and its wild progenitor *V. unguiculata* ssp. *dekindtiana*. Despite the disadvantage of this type of cross, which may be related to the identification of the loci that may be polymorphic only between more divergent genotypes, but not between more closely related genotypes, especially the ones of interest, the authors located a quantitative trait loci for seed weight that was conserved between cowpea and *V. radiata* ssp. *sublobota*. Subsequently, the second linkage genetic map developed on cowpea consisted of 181 loci, including 3 morphological markers and a biochemical marker (dehydrin) that allowed mapping of genes involved in earliness and seed weight, respectively, in linkage groups 2 and 5 [190].

16.4.5.2 Biotechnology for Resistance to Biotic Stresses

Several fungal, bacterial, and viral diseases affect cowpea at different stages of growth. The major and common diseases are anthracnose, Sclerotium stem, root, and crown rots, damping-off, Cercospora leaf spot, Septoria leaf spot, Fusarium wilt, and scab. Storage pests, *Callosobruchus maculatus* and *Calloso chinensis* cause severe damage to the cowpea seeds during storage. Transgenic cowpea has been developed for insect resistance using the bean (*P. vulgaris*) α-amylase inhibitor-1 (αAI-1) gene. Expression of αAI-1 gene under bean phytohemagglutinin promoter resulted in accumulation of αAI-1 in transgenic seeds and the recombinant protein was active as an inhibitor of porcine α-amylase *in vitro*. Transgenic cowpeas expressing αAI-1 strongly inhibited the development of *C. maculatus* and *C. chinensis* in insect bioassays [191].

16.4.6
Common Beans

16.4.6.1 Biotechnology for Tolerance to Abiotic Stresses

Abiotic stresses (climatic and edaphic) probably represent total loss in yield in common beans [192]. Estimates of area subject to phosphorus (P) deficiency suggest that 50% of bean production suffers from P deficiency, 40% may suffer from aluminum toxicity, and 73% suffers from drought. Moreover, higher temperatures and greater evapotranspiration, combined with lower rainfall, are expected to exacerbate drought in important bean-producing areas of northern Central America, Mexico,

Brazil, and southern Africa. Predictions have been made that higher temperatures in these regions will affect the altitudinal range of adaptation of bean genotypes, reducing the root growth and accelerating the decomposition of soil organic matter (mineralization) making problems of stress even more acute. However, since other bean-growing areas such as East–Central Africa and the Northern Andes will receive greater precipitation, excess rainfall, waterlogging, and associated root rots will be a problem [193].

Breeding for drought resistance in beans has a long history, in the program of EMBRAPA in Brazil [194]. Improved drought resistance has resulted from combining germplasm adapted to the dry highlands of Mexico with small seeded types from lowland Central America, through recurrent selection within each genepool. In the case of drought, roots have long been recognized as playing an important role in beans. A drought-resistant line, BAT 477, presented deep rooting under drought stress, permitting access to soil moisture at greater depths [195, 196]. However, deep rooting alone does not ensure drought resistance, and data on root density at various levels of the soil profile suggest that deep rooting genotypes are not always the best yielding materials (CIAT, 2007; CIAT, 2008). The response to drought at the physiological and molecular levels has been studied in two common bean varieties with contrasting susceptibility to drought stress (Zhu *et al.*, 2002). A number of genes were found to be upregulated (both ABA dependent and independent) in the tolerant variety Pinto Villa relative to the susceptible cultivar, Carioca, with the former displaying a more developed root vasculature in drought conditions than the latter. The International Centre for Tropical Agriculture has taken initiative for improving drought tolerance in common bean (Broughton *et al.*, 2003). The physiological studies done so far linked the intermediate drought tolerance trait in breeding line BAT 477 to greater root growth under water deficit conditions [195] and further showed that genetic control of this trait was expressed in roots and not shoots [196]. Additional genetic sources for drought tolerance were also identified in the 1980s, especially from Mexican varieties. During the 1990s, a combination of genetic sources was used to develop varieties with higher tolerance and additional mechanisms (e.g., photosynthate mobilization from leaves and stems to developing seeds) for drought–stress tolerance were identified [197]. By 2002, these drought-tolerant varieties were being tested in the field and attempts to integrate genomic techniques with traditional breeding were initiated in parallel.

16.4.6.2 Biotechnology for Resistance to Biotic Stresses

One of the most significant biotic stresses to bean crops is caused by nematodes, especially the genus *Meloidogyne*. Its occurrence is widespread and the damage is particularly striking in the case of continuous cultivation, as it occurs in center pivot-irrigated areas [198]. It is mainly controlled by the use of resistant cultivars associated with management and crop practices. The identification of resistant lines has been frequently reported in the literature [199, 200]. Common bean is exposed to *Fusarium oxysporium f sp. phaseoli* (Fop) causing wilting and early death of the plants that has been tried controlling through use of resistant cultivars [201].

The main biotechnological intervention for disease management in common bean is based on MAS. A set of microsatellites is being put together to efficiently map other populations. Linked markers were identified for the bean golden mosaic virus, anthracnose, bacterial blight, and angular leaf spot and are being used as part of a marker-assisted selection program. Other mapping populations have been developed and are being used to tag quantitative trait loci for disease and pest resistance (http://webapp.ciat.cgiar.org/biotechnology/genomics.htm).

Although a very limited work has been done using transgenic approach in common beans, a system to obtain so-called transgenic composite plants from *P. vulgaris* is available. These plants have a transgenic root system, obtained through *Agrobacterium rhizogenes* transformation of derooted seedlings [202].

16.4.7
Lentils

Lentil is a relatively small crop compared to wheat, rice, maize and soybean, ranking third among the cool season food legumes, in an harvested area of 4.08 million hectares annually, behind pea and chickpea (http://apps.fao.org/faostat). Lentils were successfully introduced to the Western Hemisphere and are now grown extensively in the United States, Canada, Chile, and Argentina, becoming an important crop and a dietary mainstay in the drier areas of the Near East and North Africa. Although mostly discontinued, lentil was a widely grown crop in southern and central Europe.

The major abiotic stresses that affect lentil are cold, drought, heat, salinity, nutrient deficiency, and nutrient toxicity. Of these stresses, drought and heat are considered the most important world wide. While the cold stress is considered important in the West Asia–North Africa (WANA) region, salinity is an important stress factor in the Indian subcontinent and to some extent in WANA. To date, progress has been made in mapping the lentil genome and several genetic maps are available that eventually will lead to the development of a consensus map for lentil. Nevertheless, marker density has been limited in the published genetic maps and there is a distinct lack of codominant markers that would facilitate comparisons of the available genetic maps and efficient identification of markers closely linked to genes of interest [203]. Comparative genomics and synteny analyses with closely related legumes promise to further advance the knowledge of the lentil genome and provide lentil breeders with additional genes and selectable markers for use in marker-assisted selection. Genomic tools such as macro- and microarrays, reverse genetics, and genetic transformation are emerging technologies that may eventually be available for use in lentil crop improvement.

16.4.7.1 **Biotechnology for Tolerance to Abiotic Stresses**
Lentil is able to produce something of value in many of the semiarid regions primarily through drought avoidance [204]. Early senescence and crop maturity forced by drought conditions are often more severe due to the usually associated high

temperatures. Molecular approaches such as marker-assisted selection may have merit; however, considerable work is needed to identify the important regions of the genome, most likely through a QTL analysis and validation of associated molecular markers.

Heat stress often accompanies drought causing difficulties in separating the two stresses and their effects on lentil growth and yield. There is general agreement that heat affects the distribution of dry matter to reproductive growth and that high temperatures have an adverse effect on lentil yields. Evaluation of the world collection of lentil germplasm has indicated that useful genetic variation is available for improving adaptation to environmental extremes [205].

Susceptibility to cold temperatures has limited production of lentil in cold highland areas of the world. However, germplasm is available that has useful degree of tolerance to cold temperatures, which makes it possible to breed winter hardy cultivars that can be planted in the fall with a reasonable expectation of surviving the winter [206]. Winter hardiness in lentil is conferred by several genes, with the combined effects of several quantitative trait loci accounting for 42% of the variation in winter survival [207]. Molecular markers associated with these QTLs, and postvalidation have potential use in a marker-assisted selection program for winter hardiness in lentil.

Although salinity problems with lentil are not widespread, it can be acute in certain regions of South Asia, the Nile Delta of Egypt, and in some areas of Turkey. Canada also has some difficulty in high salinity areas of Saskatchewan. Of the legumes, lentil is more salt sensitive compared to faba bean and soybean. Salt stress can adversely affect nodulation and N_2 fixation presumably by restricting growth of the root hairs and the potential sites of infection by Rhizobium. Some germplasm with tolerance to salt stress has been identified [208]. Future direction of lentil genomics can be summarized: (i) new marker development and fine mapping, (ii) development of new genetic materials applicable to advanced genomics, and (iii) application of advanced genomic tools for lentil genomics [209].

16.4.7.2 **Biotechnology for Resistance to Biotic Stresses**
As the case with many other legumes, rust and Ascochyta blight are the two most important foliar diseases of lentils in major lentil-producing countries. However, breeding for resistance to Ascochyta blight in lentil has been initiated only recently and is still at a very preliminary stage of large-scale screening of collections of germplasm to identify resistant resources. Multilocation testing of promising cultivars from germplasm screening coordinated by ICARDA has led to the registration of several resistant cultivars in many countries [210]. However, the recent identification of resistance genes and their relationships in several cultivated lines [211] and the confirmation of the presence of different pathotypes [212] have provided the basis to design a breeding program aimed at transferring and combining these genes. A combined bulk population and pedigree selection has been used successfully in lentil breeding at ICARDA. Nevertheless, with the development of novel techniques and the increasing understanding of the host–pathogen system, more efficient breeding methods will be applicable in breeding for resistance to Ascochyta blight in lentil [213].

Combining genes conferring resistance to different pathotypes into a single cultivar (gene pyramiding) makes it useful for diversified environments where different pathotypes are likely to be dominant. Since multiple resistance genes may have additive effect, and even if it is not, the presence of more genes implies that pathotypes have to be virulent to all the genes before a resistant cultivar loses resistance [214]. This procedure of combined resistance has been adopted at ICARDA for improving resistance to Ascochyta blight of chickpea [215]. This method can also be used in breeding for lentil Ascochyta blight resistance as different pathotypes and pathotype-specific resistance have been identified [216]. Genes for Ascochyta blight resistance have been identified in wild lentil species, and hence transferring these from wild species into elite cultivars will be an important approach in breeding for resistance. Ford *et al.* [217] identified seven RAPD markers linked to the resistance locus conferring Ascochyta blight resistance in "ILL 5588" using bulk segregation analysis. Five of the seven RAPD markers were within 30 cM of the resistance locus and the closest flanking markers were approximately 6 cM and 14 cM away from the resistance locus.

Although not a routine, genetic transformation in lentil has been feasible in last few years [218]. Therefore, production of transgenic lentil plants and consequently the application of transgenic techniques in lentil breeding may soon become important for the genetic improvement of this legume.

16.5
Future Prospects

The exploitation of the genetic and genomics resources and biotechnological interventions already employed in model legumes can be a well adopted biotechnological approach for grain legume improvement. Since *Medicago truncatula* is already being studied to unravel resistance to a large number of pathogens, from parasitic plants, bacterial pathogens, and nematodes to fungal and oomycete pathogens. Hence, the transcriptomic and proteomic approaches developed for this model legume can be used to understand the molecular components and identify candidate genes involved in defense against these pathogens for the cool season legumes [219]. Besides, soybean can serve as model for the many crop species in the Phaseoleae due to its vigorous studies being undertaken for nodulation, mycorrhization, and plant-symbiont signaling [220].

16.6
Integration of Technologies

Although significant efforts were made in the past to adapt the plant to the environment, the emerging concept is to genetically tailor the crops for maximizing resource-capture efficiency, yield, and yield stability. The genomics-assisted crop breeding offers unprecedented opportunities to identify major loci influencing

the target traits and to select for plants with the desirable combination of alleles via marker-assisted selection, marker-assisted backcrossing (MABC), or marker-assisted recurrent selection (MARS). Similarly, the current and fast emerging technologies such as RNAi technology, targeted gene replacement using zinc finger nucleases, chromosome engineering, MARS and GWS, NGS, and nanobiotechnology should be utilized in an integrated way to combat the adverse conditions in legume crops. Although the advances in biotechnology greatly facilitate grain legume improvement, a more comprehensive knowledge of resistance or tolerance mechanisms is required to direct breeding in these crops. 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. Similarly, genetic engineering options for targeting of transgene expression to particular conditions (e.g., using stress-responsive or tissue-specific promoters) can be potentially integrated with proteomic and metabolomic approaches for monitoring the effect of the transgene in order to be able to take advantage of the knowledge being gathered from "omic" technologies. Overall, for biotechnology to fulfill its potential for grain legume breeding, there need to be a synergy between classical breeders and biotechnologists to first ensure that the tools of biotechnology are applied to the most pressing and appropriate problems and, second, to ensure that pathways for delivery/uptake into breeding programs are in place. However, the ultimate objective of effective utilization of the genomic resources, identification of suitable genes for candidate gene-based association mapping and/or to be used as transgenes could only be possible through an integrative approach taking the classical breeding and biotechnological approaches together.

16.7
Conclusion

Genetic diversity is critical for any successful breeding program and genetic resources are important sources of such diversity as well as of traits that permit continued yield increases under climate change scenarios as materials may have evolved under some of the harshest conditions [221]. Despite the known fact that the wild germplasms for different crops have found to be a rich source of resistance to these constraints, introgression of genes for resistance or tolerance into cultivars are a cumbersome jobs due to bottlenecks in crossability. Hence, the present-day agricultural research programs require focus on an integrated genetics and genomics approach to dissect molecular processes from transcriptome to phenome. Where diversity is lacking for critical traits in cultivated species, tapping wild relatives can be employed through wide hybridization to create novel diversity in polyploidy species [222]. Moreover, with the improvisation through conventional breeding approaches, different molecular breeding approaches are also being used to accelerate utilization of the substantial variability among the grain legume

landraces and germplasm lines for various morphological, physiological, and agronomic traits.

However, challenges such as nonavailability of linkage data, low levels of DNA polymorphism within the primary (cultivated) gene pool, and availability of lesser number of molecular markers pose considerable restrictions and hence genome sequencing initiatives would raise newer genetic and genomic resources in grain legumes.

Moreover, transgenic technology is ought to be a possible solution negating constraints to grain legume productivity and improvement, widening the chance of introducing the transgenes for resistance or tolerance to cultivated varieties without compromising their yield potential. Identification of novel promoter and enhancer elements will also be critical to achieving efficacious expression of antifungal/ antimycotoxin genes in grain legumes. In addition to nuclear transformation, development of plastid transformation protocols will enable high-level expression of multiple resistance genes in the transgenic crop development reducing the chances of out crossing.

Grain legume crops are genetically related and therefore exhibit synteny at the genetic and genomic levels and consequently functional similarities at physiological and phenotypic levels. Hence, cross-crop learning has tremendous potential for understanding of genetic and physiological mechanisms and control points for disease and pest resistance, drought and other stress adaptation, nutritional quality, biological nitrogen fixation, and other key traits. Clearly, modern biotechnological interventions have the potential to contribute to more productive and stable grain legume farming systems with increased productivity and income and improved health and resilience to climate change.

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