Chapter 6

Functional Genomics of Biotic and Abiotic Stresses in *Phaseolus vulgaris*

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Additional information is available at the end of the chapter

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Abstract

Common bean is the most important legume for human consumption in the world, being a crop extremely diverse in cultivation methods, uses, range of environments in which it is adapted, morphological variety, among others. Besides its high demand and production, this crop is threatened by a series of biotic and abiotic adversities during its life cycle, which leads to losses in yield of up to 100%. In this chapter, we explored the main constraints that affect common bean and the ways this plant reaches tolerance or resistance to them, highlighting studies at the molecular level that enabled to understand the mechanisms by which common bean perceives, responds, and adapts to a stress condition. Special focus has been given to the most recent findings in the understanding of the mechanisms underlying drought tolerance and anthracnose resistance. Thereby, we reviewed some genetic and functional genomic studies concerning the genes and pathways involved in each case. Furthermore, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

Keywords: Common bean, anthracnose, drought, genetic resources, gene expression

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important legume crop for consumption worldwide [1]. It is cultivated in a range of crop systems and environments, being Latin America the leading producer and consumer, where beans are a traditional and significant food source, especially in Brazil, Mexico, the Andean Zone, Central America, and the Caribbean [2]. As a source of protein, folic acid, dietary fiber, and complex carbohydrates, common

beans are considered nutritionally rich and when consumed as part of the diet can lead to an increase in the use of maize and rice proteins since their amino acids are complementary [2]. They are also a good non-meat source of iron, providing 23–30% of the daily recommended levels of this element in a regular adult diet [2–3].

In Latin America, Africa, and Asia, common bean is primarily a small farmer crop cultivated with few purchased inputs and is subject to a large amount of biological, edaphic, and climatic issues [2–4]. Conditions under which common beans are regularly cultivated in this regions are extremely variable [3], and such factors coupled with the highly specific local preferences for seed characteristics (size, shape, color) have been challenging to establishing the breeding strategies in accordance with what is needed.

Beans from these regions usually present low yielding [2], since they are frequently cultivated employing low to non-mechanized irrigation systems. Common bean is mostly grown in drought-prone areas, and long-term drought exposure periods seems to be a global and endemic threat affecting the majority of the production areas [4]. It has been observed that common bean is particularly susceptible to drought especially during the flowering and grain-filling stages (R5 and R8, respectively) [5, 6]. Moderate levels of water deficit usually lead to a reduction in plant biomass, lower seed number per pods, earlier maturation, lower seed yield and weight, and reduction in nitrogen fixation [7].

Not only abiotic factors but also several biotic constraints represent a significant threat to common bean cultivation. Fungi, bacteria, viruses, and nematodes cause a series of diseases, concurring for the death of some plants or even significant areas from whole plantations, causing a severe reduction in yield. Examples of such diseases are rust, white mold, anthracnose, root rots, bacterial blights (halo, yellow, common), powdery mildew, mosaic viruses, etc. Environmental conditions (temperature, soil moisture) and management practices (varieties, crop rotation, irrigation, and chemical control) may prevent the establishment of some diseases and reduce losses, but for some of them the most appropriate strategy for controlling consists on the development of resistant varieties and high-quality seeds.

This chapter is especially driven to describe the most recent developments in the understanding of the molecular mechanisms involved in drought tolerance and anthracnose resistance. In that purpose, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

2. Genetic resources

2.1. Center of origin and domestication of common bean

Beans belong to the Fabaceae family (Leguminosae, Papilionoidae) and genus *Phaseolus*. About 55 species of *Phaseolus* are described but only five are cultivated: *P. vulgaris*, *P. acutifolius*, *P. lunatus*, *P. polyanthus*, and *P. coccineus* [8].

P. vulgaris is naturally distributed in a wide area from northern Mexico to northeastern Argentina. High morphological diversity has been found among wild populations of *P.*

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vulgaris from one to the other extreme of the geographical distribution of the species [9, 10]. This variability is observed in different leaf shapes, growth habits, flower colors but especially for seeds in terms of colors, shapes, and sizes [10]. This variability has also been observed at the molecular level, with several molecular marker studies such as with microsatellites [11–15], AFLP [14–16], and SNPs [17–20].

Several of these studies recognized two major ecogeographical gene pools of wild beans: Mesoamerican and Andean. However, the geographic structure of the wilds reveals more complexity, with an additional third pool between Peru and Ecuador, characterized by a particular storage seed protein, phaseoline type I [21, 22]. Further examinations showed wild populations from Colombia to be intermediates. A marked geographic structure in populations from the Mesoamerican pool has also been described [23, 24]. Originally, the population from northern Peru and Ecuador was considered an ancestral population from which *P. vulgaris* originated. From this core location, beans probably were spread north and south, resulting in the Mesoamerican and Andean pools, respectively [22, 25, 26].

Nevertheless, based on several studies [27–29], there has been a discussion over an alternative and older hypothesis which considers that ancestral beans were distributed through Mesoamerica. The high genetic diversity encountered within these gene pools has been used to support this hypothesis. Furthermore, the Mesoamerican origin of the common bean has been suggested based on sequence analysis of data from five small gene fragments [32]. A whole-genome comparison among 30 individuals from each Mesoamerican and Andean wild populations showed high genetic differentiation among gene pools and, a demographic inference for the Andean gene pools, suggested it was derived from a Mesoamerican population with only a few thousands of individuals [20]. Nevertheless, the debate on the origin of the species remains and more studies are on their way to better understand the core center of origin of common bean.

Likewise, the domestication process of *P. vulgaris* has been another matter of debate and extensive molecular studies. Initially, morphological and enzyme profiles showed the existence of two major centers of bean domestication: Mesoamerica and Andean, encompassing six races [10]. There are indications that nearly 8,000 years ago common bean was independently domesticated in Mexico and South America [30–33]. Domestication was followed by local adaptations resulting in landraces with different characteristics [20]. However, much more has yet to be deciphered and the recent application of genomic approaches is promising to a better understanding of the domestication processes of common bean and other crops [34].

2.2. Core collections

The high diversity of common bean has been collected in germplasm banks in which those are not only kept but also constantly improved, generating new genetic materials by adding new combinations obtained through many crosses and new generated populations. Several bean germplasm collections are available, but some of the core collections that must be highlighted here are held at the Centro Internacional de Agricultura Tropical (CIAT), in Cali, Colombia. Information on every wild and domesticated beans from this collection may be obtained in the website http://isa.ciat.cgiar.org/urg/main.do?language=en. Another core collection is from the

United States Department of Agriculture (USDA), found on http://iapreview.ars.usda.gov. Brazil has held a very significant collection of landraces and domesticated beans at EMBRAPA Arroz e Feijão and also at the Agronomic Institute of Campinas, which has been developing several new commercial varieties (http://www.iac.sp.gov.br/areasdepesquisa/graos/feijao.php). Much more details about bean collections are found on Genesys (https://www.genesys-pgr.org/welcome), a portal to information about Plant Genetic Resources for Food and Agriculture, describing many bean accessions and the places where they are kept. These collections comprise a very rich source of genetic materials that possess several features to be exploited in functional genomic and molecular breeding studies for the species. Among the genetic resources available are wild beans, landraces, breeding lines, recombinant inbred populations, all distinguished between the Andean and Mesoamerican gene pools.

2.3. *Phaseolus vulgaris* – The genome

A recent publication showed the work that has been done for many years to sequence the genome of the common bean, whose assembly has been made public by a consortium between the USDA-NIFA project "A sequence map of the common bean genome for bean improvement" and DOE-JGI and ARRA (Phaseolus vulgaris v1.0 – http://phytozome.jgi.doe.gov/). In total, 472.5 Mb of the 587-Mb genome were assembled and 98% of the sequence were genetically anchored on the 11 chromosomes, using a SNP high-density map (7,015 markers) genotyped in the RIL (recombinant inbred lines) population derived from the cross Stampede × Red Hawk and another map with 261 SSRs and a set of Infinium markers. The 472.5 Mb were arranged in 41,391 contigs (~9.32% gap) and the annotation revealed 27,197 total protein-coding genes and 31,638 protein-coding transcripts, resulting in 4,441 total alternatively spliced transcripts [23]. The publication of this genome opened a series of new resources for developing research in many fields such as the mechanisms involved in biotic and abiotic stresses in common bean.

3. Identification of genes involved with anthracnose resistance

The pathogenic system *Colletotrichum lindemuthianum*/*Phaseolus vulgaris* has been studied as a model for almost one century [36] and, its infection mechanisms and disease development were extensively studied in the 1980s [37, 38, 39]. This species of *Colletotrichum* is one of the most studied due to its economic importance, infection strategy [38], ease of *in vitro* cultivation [40], and availability of an efficient and reproducible transformation system [41]. As a model system for plant/fungi interaction, it can provide valuable information in several aspects, like plant defense responses, phytoalexins, fungal-degrading cell wall enzymes, differentiation of fungal infection structures.

The susceptible common bean cultivars establish an interaction of compatibility with this fungus, what allows the development of the anthracnose disease, strongly affecting production and yield of beans; furthermore, this fungus has great variability and many races identified [42, 43]. With this, the genetic resistance is an important way of disease control. Genetic studies

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indicate that the common bean resistance to the anthracnose is related to multi-allelic loci [44, 45], which mostly comprise dominantly inherited genes denominated *Co* [45]. Bean cultivars resistant to anthracnose containing *Co* gene (s) respond to pathogen inoculation with an incompatible interaction. This interaction initiates with the pathogenic fungus inoculation, causing physiological variations and rapid changes in gene expression that activate defense responses in the host plant. Necrotrophic points, typical of a hypersensitive reaction (HR), occur at the infection site, resulting in a limited fungal growth. The HR, considered the primary response of the plant to the pathogen attack, is characterized by an oxidative burst due to the formation of reactive oxygen species (ROS) [46]. This initial plant response can be considered definitive in the determination of resistance to the pathogenic agent.

In the compatible interaction, the establishment of the pathogen in the plant tissue is aided by the production, by the fungus, of virulence effectors induced by the host [47, 48]. The life strategy adopted by the fungus (hemibiotrophic) make infected tissues remain without outward symptoms for up to three or more days [49, 50], and only after the entrance in the necrotrophic phase cause plant cell death and emergency of pathogenic lesions.

Despite the multi-allelic resistance already described for the common bean, new sources of resistance should always be searched due to the high variability among pathogen populations and occurrence of newly evolved virulent races. Furthermore, knowing the molecular pathways involved with the process of resistance in the plant can enable the transference of important genes to susceptible cultivars.

Common bean is not a species prone to be genetically transformed, although there is already a transgenic cultivar resistant to the Golden Mosaic Virus [51]. Furthermore, the genome of common bean was made available only recently, and reverse genetics through the use of mutant lines is still difficult due to few resources. Then, transcriptomic analysis appears as a suitable method to investigate the changes in gene expression in a plant under any kind of stress.

3.1. Gene expression profiles from an incompatible interaction

Studying gene expression profiles of incompatible interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum* may be an advantageous strategy to identify genes involved with anthracnose resistance because it can provide a direct answer about the potential modulations occurring in metabolic processes during an infection event with a resistance response by the host.

The first study devoted to generate a unigene data set of common bean using ESTs sequencing was described by [52], through the analysis of three EST libraries from the cultivar SEL 1308, consisting of 19-day-old trifoliate leaves, 10-day-old stem shoots, and 13-day-old stem shoots inoculated with the race 73 of *C. lindemuthianum* in an incompatible interaction. At that time, a total of 5,255 ESTs were sequenced, 2,332 from inoculated stem shoots, with 1,583 unigenes assigned for this library. More recently, [53] used this database to select candidate genes based on the number of ESTs found per unigene (or tentative contig) in each library, to study expression profiles in temporal and spatial scales during fungus infection. Twelve genes were

chosen and tested in leaves, hypocotyls and epicotyls inoculated with *C. lindemuthianum* (Figure 1).

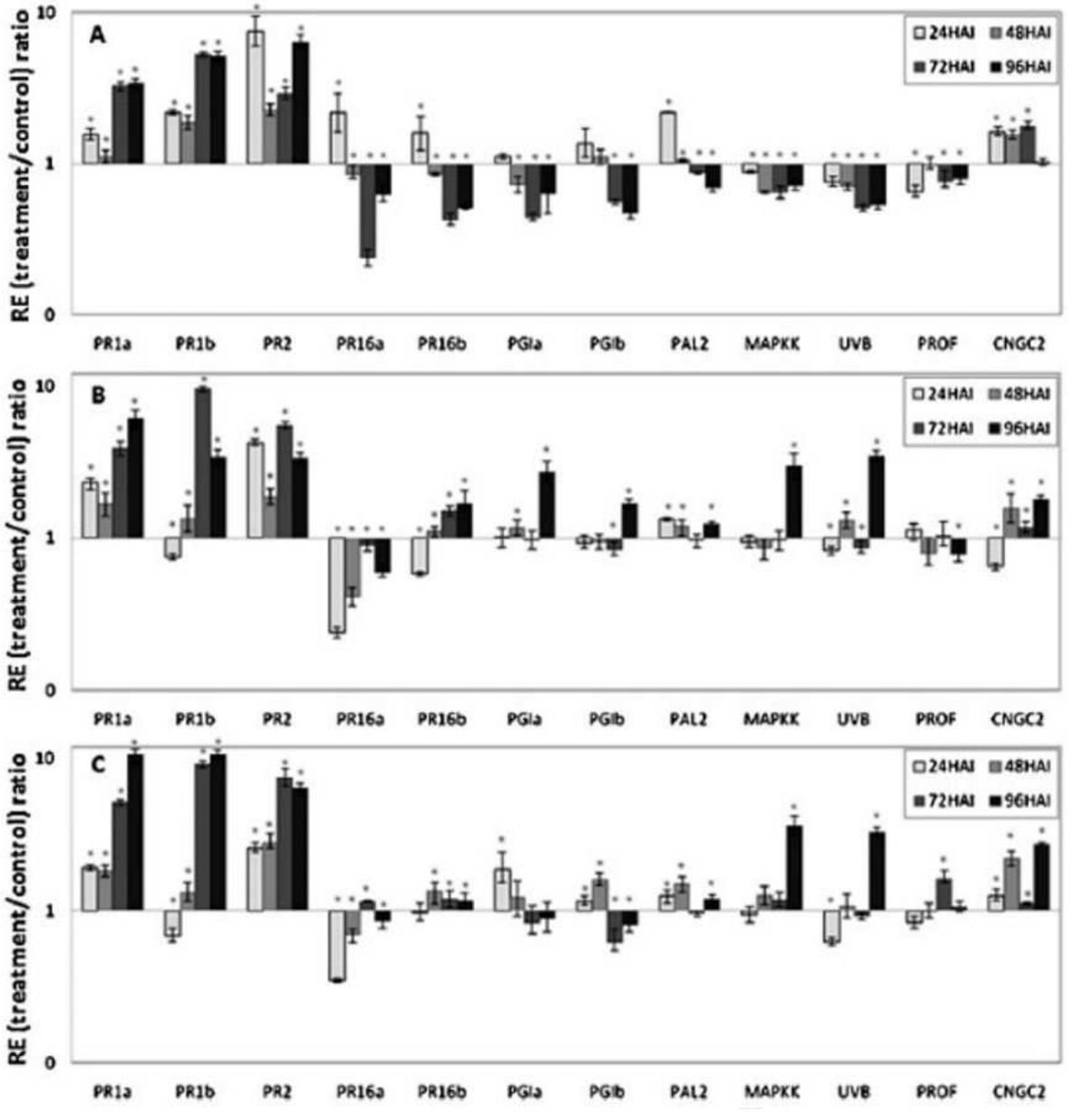

Figure 1. Relative expression (RE) ratio of 12 pathogenesis resistance-related transcripts in leaves (A), epicotyls (B), and hypocotyls (C) of common bean genotype SEL 1308 at 24, 48, 72, and 96 HAI with the race 73 of *C. lindemuthiaum*. Non-inoculated tissue was used as control for expression levels to determine the RE ratio. The symbol * above the bars indicates statistical significance calculated using the Pair Wise Fixed Reallocation Randomization Test with $P \leq 0.05$. (Extracted from [53]).

All genes showed modulation during this incompatible interaction. Some of them were rapidly activated and kept this activation, like PR1a, PR1b (known as good molecular markers for SAR (systemic acquired resistance)), and PR2 (a b-1,3-glucanase) (Figure 1), which act in plant

defense by hydrolysing the cell walls of the fungal pathogens. All the others showed a variety of expression patterns according to time and tissue, for instance, PR16 proteins (germin-like), which were upregulated early in leaves and then fall down, and in epicotyls and hypocotyls only PR16b was upregulated in late periods of analysis (Figure 1). This kind of study not only give us an idea of the kinetics of induced defense responses of common bean against the anthracnose fungus but also can be used as a base line for others studies of resistance against a broad range of pathogens [53]. Furthermore, this work revealed differential and specific transcriptional profiles in different tissues of common bean, where specific defense processes may occur to contain the development of a pathogen. For more details, see [53].

3.2. The immune system model for *Phaseolus vulgaris*/ *Colletotrichum lindemuthianum*

The innate immunity is a primitive way of defense against microbial infection shared by plants, insects, and animals. Differently from mammals that have mobile cells specialized in defense, each plant cell is responsible for its own defense. Thus, each cell integrates environmental signals in order to activate local and systemic defense responses.

The same EST libraries described before [52] were used by [54] to investigate global changes in gene expression of *P. vulgaris* inoculated with *C. lindemuthianum* in an incompatible interaction. In an extensive bioinformatics analysis, the ESTs were aligned by tBLASTX with the *Arabidopsis thaliana* (L.) Heynh genome, which is completely annotated and curated. With this, it was possible to conduct a functional comparison between the fungus-inoculated and the mock-inoculated library. Figure 2 shows the overall mechanisms found in this study. It was found that some processes involved with plant–pathogen interaction were upregulated in common bean in response to the presence of fungus, like defense response to fungus (GO: 0050832), regulation of defense response GO:0031347), regulation of response to stress (GO: 0080134), and stomatal movement (GO:0010118).

Response to cytokinin stimulus (GO:0009735) and ethylene-mediated signaling pathway (GO: 0009873) were upregulated, while jasmonic acid biosynthetic (GO:0031408) and metabolic (GO: 0009694) processes, as well as response to gibberellin stimulus (GO:0009739) and abscisic acid-mediated signaling pathway (GO:0009738) were downregulated, indicating that there may be a hormonal control and cross-talk in common bean defense against *C. lindemuthianum*. According to [54], hormonal mechanisms can be used in some pathosystems for resistance and in others for susceptibility depending on the fungus life-style. While jasmonates (JA) were found to be important in disease susceptibility in Arabidopsis and tomato infected with *Pseudomonas syringae* [55, 56], a biotrophic bacterium, in common bean it is not used in signaling since *C. lindemuthianum* is a hemibiotrophic pathogen.

Still based on the analysis of ESTs libraries, infected common beans have its metabolism modulated for detoxification from ROS burst, once HR is occurring during the incompatible interaction; also, a downregulation of genes was observed related to plant development (organelle fission (GO:0048285), cell cycle process (GO:0022402), pattern specification process (GO:0007389), post-embryonic morphogenesis (GO:0009886), and regulation of post-embryonic development (GO:0048580), typical of plants under stress that needs to reallocate resources to defense responses.

Finally, transcripts encoding for cell wall proteins showed an increase in abundance, suggesting that activities as cell wall modification, pathogen recognition, and transport and secretion of defense compounds are important in bean defense against anthracnose.

When looking for molecular components of the plant innate immunity (PTI – PAMP-triggered immunity or ETI – effector-triggered immunity), [54] observed that ETI (characterized by HR) can negatively regulate PTI. Transmembrane receptor protein tyrosine kinases and MAPKKK/MEKK transcripts were significantly downregulated in fungus-inoculated library and this data validate by RT-qPCR.

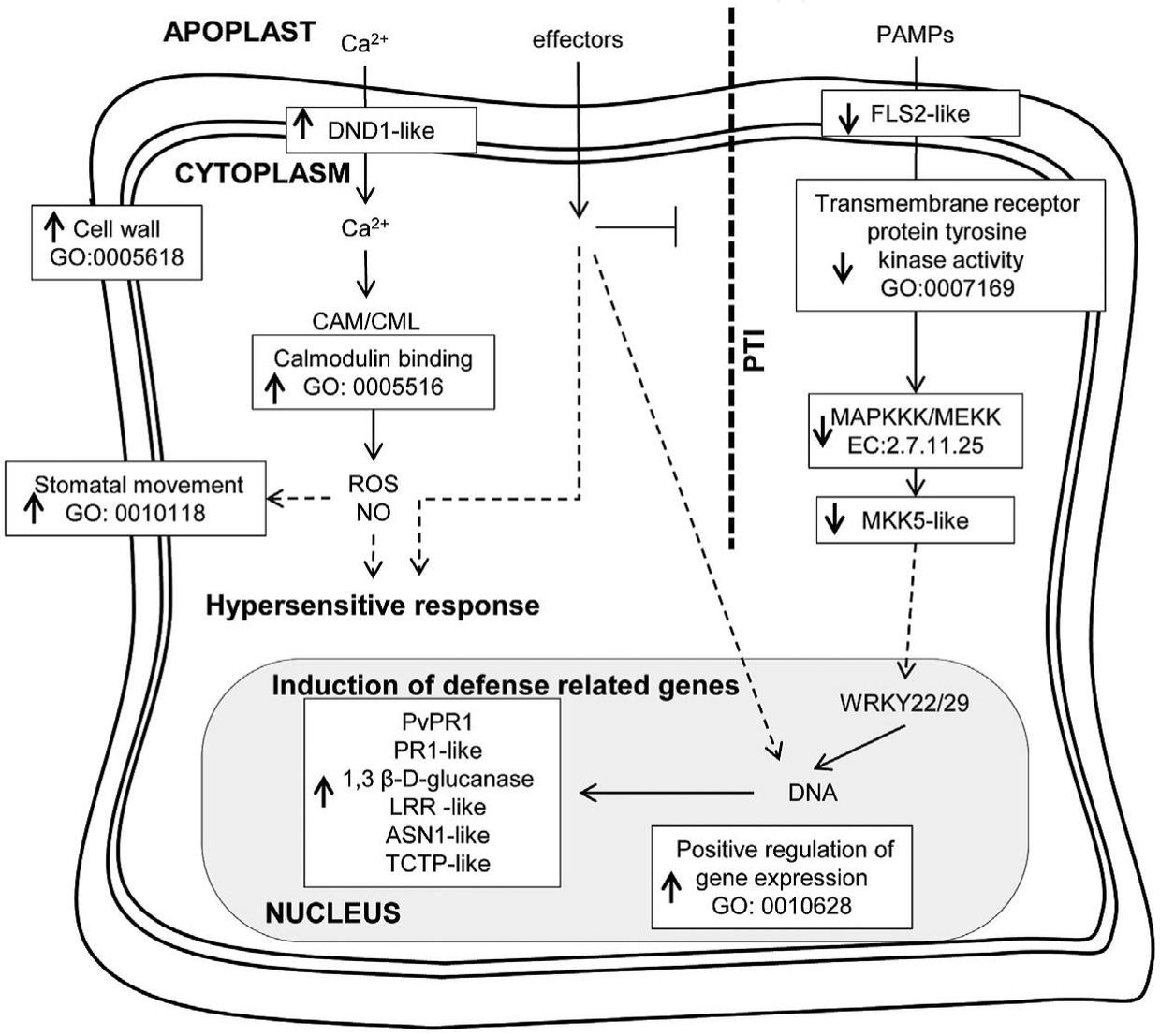

Figure 2. A model of the bean innate immune system. The proposed model represents key molecular components and metabolic processes known to be involved in plant–pathogen interactions. Gene Ontology (GO) categories and Enzyme Codes (EC) inside boxes are differentially represented in the bean EST libraries (arrowheads pointing down represent downregulation and arrowheads pointing up represent upregulation). Continuous arrows represent established relationship between components of the pathway and intermittent arrows represent undirected relationship. Components of PAMP-triggered immunity (PTI) are depicted to the right of the diagram. (Extracted from [54]).

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4. Identification of genes involved with drought tolerance

4.1. Gene expression profiles from Subtractive Libraries of cDNA and RT-qPCR

Long-term global climate changes have conducted to an increase in the occurrence of drought episodes in different locations around the globe [57, 58]. This fact concurrently with agriculture expansion into marginal areas have led to increasing environmental instability, a limiting factor for crop yielding with potential negative impact on food stocks worldwide. This problem is especially aggravated by the rapid human population growth and consequent augmented food demand, especially in developing countries. Therefore, drought has been considered one of the main abiotic constraints that affect agriculture [59].

Plant responsiveness to drought stress can be affected by different factors; it mainly depends on the severity of the event, including the extension of the water-deficit period, and if the plant has already been exposed to a previous regime of acclimatization to this condition [60]. Acclimatization to drought results from a series of integrated events that comprehend the perception of the stress by the plant, translation of the signal, the regulation of the expression of specific genes, and the consequent shifts at metabolic level [61].

Drought perception often leads to a reduction in the photosynthetic rates of the plant, affecting its growth, which is directly related to shifts in carbon and nitrogen metabolism [62]. This reduction on the photosynthetic net is a result of a series of coordinated events such as stomatal closure and the reduction on photosynthetic enzymes activity [63, 64]. At cellular level, drought stress results in the accumulation of the chemically reactive molecules containing oxygen termed as ROS (reactive oxygen species), which ultimately can also drive to the oxidative stress of the photosynthetic apparatus [65,66], thus ROS-efficient removal for avoid oxidative stress can be used as a measure for drought stress tolerance in plants [67]. These molecules act inside cells as secondary messengers involved in signaling transduction that leads to specific stress responses [65]. At molecular level, some specific sets of genes can undergo different processes of regulation of their expression (mainly through cycles of induction and repression of expression) determining new protein synthesis profiles, therefore changing their biological functions [61]. Several genes have been both collectively and individually implicated in drought stress response in plants, but the identification of which ones would be more useful for adoption at breeding and transformation approaches aiming the improvement of drought stress tolerance remains a great challenge [68, 69].

Strategies for plant transformation and genetic breeding usually focus on the transfer of a single or a small set of genes that can codify for specific biochemical pathways or for final targets of the signal transduction pathways that usually are controlled by constitutively active promoters [70]. These gene products protect the plant against the damages caused by drought stress and are divided into different classes: osmoprotectors (amino acids, dimethyl-sulfonyl compounds, mannitol, sorbitol, complex carbohydrates); enzymatic and non-enzymatic ROS scavengers; LEA proteins; heat-shock proteins; ion transporters; fatty acid desaturases; aquaporins; signaling components (homologous to histidine kinases, MAP kinases, Ca^{+2}-dependent protein kinases, protein phosphatases, Ca^{+2} sensors, inositol kinases); transcription

factors (EREBP/AP2, bZIP, ABRE, NAC, MYB); and growth regulators (ABA, cytokines, brassinosteroids) [60–71, 72].

At the transcriptional level, expressed sequence tags (EST) sequencing has been widely used to discover and identify genes potentially involved in drought stress response [73, 74]. Therefore, by using a great amount of transcriptome profiling methods, researchers are being able to contrast genotypes with different potential for drought tolerance, thus increasing the already large datasets of candidate genes for using in studies regarding the improvement of drought stress in plants.

Suppressive subtractive hybridization (SSH) method has been successfully used to construct cDNA libraries enriched in transcripts that are differentially expressed in target tissues, developmental stages, and specific treatments in various biological systems [74,75]. The SSH method [76] consists on the hybridization of one cDNA population (*tester* – sample whose genetic profile is of interest, e.g., drought-tolerant genotype), with an excess of cDNA from a control population (*driver* – usually drought-susceptible genotype or well-watered control), followed by the separation of the nonhybridized molecules (*target genes* – the ones of interest) from the hybridized ones (what is common for both samples). In this session, we are aiming to present some of the results obtained by our group during the construction of a SSH library contrasting populations of cDNAs extracted from root tissues of two common bean genotypes, BAT 477 (tester – drought-tolerant) and Carioca 80SH (driver – drought-susceptible), both submitted to a 192 hours of water-deficit regime at the R5 developmental stage [77].

The sequencing of the SSH library consisting of a BAT 477 cDNA population enriched for transcripts exclusively expressed by this drought-tolerant genotype under 192 hours of water-deficit generated 1,572 valid reads that were grouped into 189 contigs and 931 singletons (total of 1,120 unigenes). Public green plant EST databases (available at the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/) and bioinformatics tools were used for initial trimming, clustering formation, gene annotation. Final functional annotation was achieved using the Gene Ontology Consortium database (http://geneontology.org/) combined to the CS model (*CombinedScheme*) developed by [78] (http://www.biochem.ucl.ac.uk/~rison/FuncSchemes/) (for further details on adopted bioinformatics tools and analysis specifications, see [77]).

Gene annotation based on homology search using the BLASTX tool and redundant sequences with E-value ≤ e-5 generated putative information on 896 reads: 315 reads displayed similarity with sequences with not yet assigned putative or hypothetical functions, and 259 reads had good quality control but had no similarity with sequences available in public databases. Table 1 lists the most abundant contigs annotated via BLASTX tool and classified under the biological process that they might be involved in the plant. Final functional annotation classification of the 896 reads is summarized in Figure 3. The six main functional classes are described as follows: 1. Cellular Metabolism (Energy, Macro/ Micronutrients); 2. Biological Process (Cell Division, Regulation, Signaling, Cell Death, Signal Transduction, and Nuclear Cycling); 3. Transport of Compounds; 4. Structural Organization (Membrane, Cell Wall, Nucleus, Organelles, and Nodules); 5. Information Pathways (DNA, RNA, proteins, and transposons); and 6. Stress Response (Biotic and Abiotic Stresses).

Access code in library	Number of reads	GI number	Description/ Species	e-value
Cellular Metabolism (Energy/Micro and Macromolecules)				
Contig147	3	|255579310|	pyruvate decarboxylase, putative [Ricinus communis]	4e-80
Contig7	3	|83283965|	malate dehydrogenase-like protein [Solanum tuberosum]	e-171
Contig23	3	|255638912|	glyceraldehyde-3-phosphate dehydrogenase [Glycine Max]	e-119
Contig28	3	|255540625|	glutaredoxin-1, grx1, putative [Ricinus communis]	2e-40
Biological Processes				
Contig123	3	|224094081|	spliceosomal complex, [Populus trichocarpa]	3e-35
Contig171	3	|75304713|	Methionine adenosyltransferase, [Phaseolus lunatus]	1e-83
Contig79	4	|156181612|	S-adenosylmethionine decarboxylase [Phaseolus vulgaris]	3e-25
Contig127	4	|75304713|	Methionine adenosyltransferase, [Phaseolus lunatus]	5e-90
Abiotic Stress Response				
Contig74	4	|42571665|	interferon-related developmental regulator family protein [Arabidopsis thaliana]	6e-53
Contig105	3	|192910730|	light-inducible protein ATLS1, [Elaeis guineensis]	2e-30
Contig14	3	|75708857|	group 3 late embryogenesis abundant protein, [Phaseolus vulgaris]	6e-23
Contig61	3	|806310|	proline-rich protein, [Glycine max]	7e-18
Contig37	4	|1732556|	LEA5 [Glycine max]	3e-34
Contig97	4	|1350522|	LEA protein [Picea glauca]	3e-27
Contig24	9	|1732556|	LEA5 [Glycine max]	3e-34
Biotic Stress Response				
Contig3	3	|184202203|	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig3	3	|184202203|	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig17	9	|130835|	PvPR2 [Phaseolus vulgaris]	1e-79
Transport				

Access code in library	Number of reads	GI number	Description/ Species	e-value
Contig164	3	|61651606|	plastidic phosphate translocator-like protein1 [Mesembryanthemum crystallinum]	1e-61
Contig80	4	|255587991|	cation:cation antiporter [Ricinus communis]	1e-39
Contig2	3	|255552798|	ATP binding protein, putative [Ricinus communis]	8e-30
Contig64	4	|255637247|	calcium ion binding [Glycine max]	2e-38
Structural Organization (Membrane, Cell Wall, Nucleus, Nodulation and Organelle)				
Contig142	3	|255549412|	Vesicle-associated membrane protein, putative [Ricinus communis]	8e-31
Contig137	3	|146233385|	abscisic acid ABA receptor [Populus trichocarpa]	1e-24
Contig148	3	|194466205|	putative L24 ribosomal protein [Arachis hypogaea]	2e-23
Contig11	5	|255584772|	histone h2a, putative [Ricinus communis]	2e-27
Contig19	3	|57013900|	NitaMp027 [Nicotiana tabacum]	6e-33
Contig83	4	|30682545|	ARF3 (ADP-Ribosylation factor 3) [Arabidopsis thaliana]	1e-59
Information Pathways (Processing of DNA, RNA and proteins/ Transposons)				
Contig154	3	|187940303|	NAC domain protein [Glycine max]	8e-84
Contig51	4	|20138704|	eIF-5A [Manihot esculenta]	7e-40
Contig52	4	|255646048|	transferase activity [Glycine max]	2e-58
Contig162	3	|155212489|	N3 protein [Glycine max]	1e-47
Unclassified				
Contig72	3	|255626205|	unknown [Glycine max]	3e-78
Contig87	3	|255639776|	unknown [Glycine max]	3e-71
Contig98	3	|255647862|	unknown [Glycine max]	8e-55
Contig145	3	|255646578|	unknown [Glycine max]	5e-47
Contig6	4	|224101339|	predicted protein [Populus trichocarpa]	5e-30
Contig64	4	|255637247|	unknown [Glycine max]	2e-38
Contig77	4	|255637264|	unknown [Glycine max]	2e-10
Contig82	6	|255629893|	unknown [Glycine max]	7e-27

Table 1. List of most abundant contigs containing the original ID of SSH library, number of reads assigned, NCBI identification number (GI) of the EST used for gene putative annotation inference, EST description and correspondent species, e-Values. ESTs are organized according to the functional class Biological Process [77].

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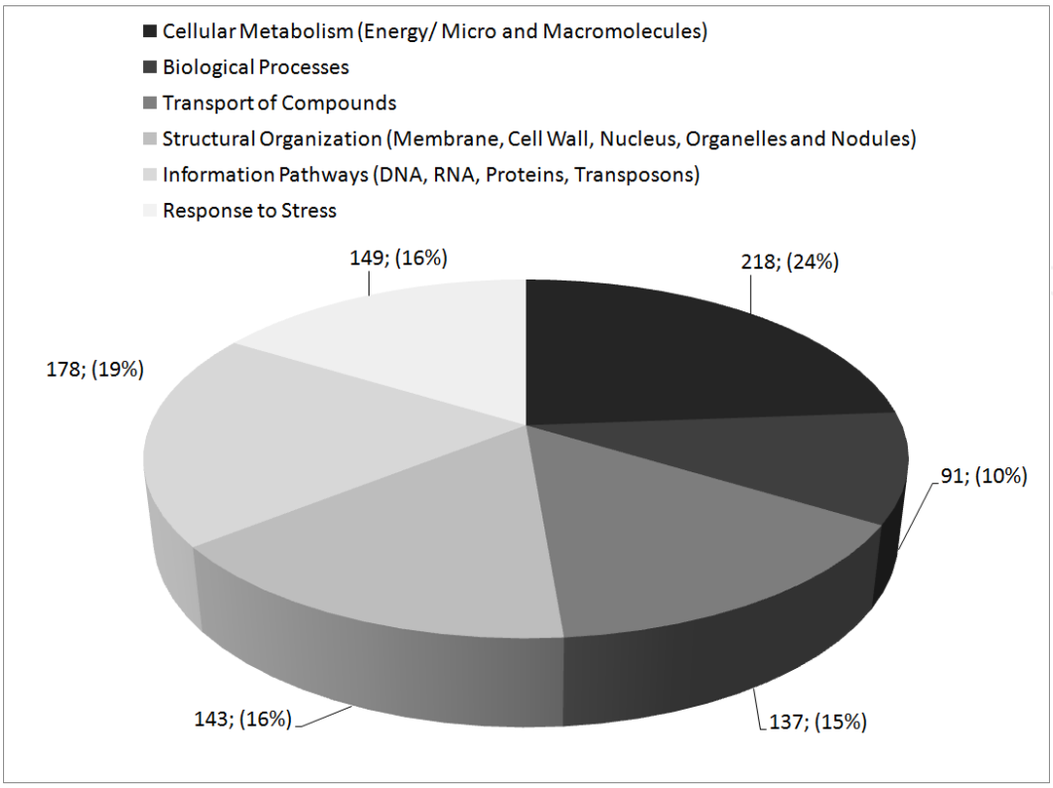

Figure 3. Final functional annotation classification of the 896 reads with positive putative gene description information (E-value ≤ e-5) obtained after blastx homology search using NCBI EST green plant public database (http://www.ncbi.nlm.nih.gov/). Each sector contains the relative EST numbers, in parenthesis the representation percentage in relation to the total number of ESTs successfully annotated. (Extracted from [77]).

The most abundant functional class was Cellular Metabolism (218 ESTs), something that was already expected since, as mentioned before, plants that undergo long periods of water deprivation tend to reduce its photosynthetic rates due to shifts in carbon and nitrogen metabolism, therefore needing to adjust its basal metabolic rates in order to keep homeostasis. Such elevated number of ESTs may be related to a more efficient mechanism of metabolic adjustment present in the drought-tolerant genotype BAT 477 that allows these plants to better adapt during the drought period, thus achieving better survival rates. And, 148 reads were grouped at the Response to Stress and some of them may be directly linked to drought stress tolerance: transcription factors (NAC, DREB, ABRE, WKRY, bZIP, MYB), transmembrane transporters like aquaporins, K^+/H^+ pumps and Ca^{+2} transporters, osmoregulators (LEA proteins, dehydrins, proline-rich peptide chains), and proteins associated with protection (heat-shock proteins, chaperones) and degradation (ubiquitins) [77].

A common bias usually associated with the SSH library construction technique combined with the traditional Sanger-based sequencing technique [79] is the possibility of obtaining false-positives. Recently, the use of SSH library technique combined with new high-throughput NGS-sequencing technologies [74–80, 81] has provided evidence for solving this issue since they are more able to achieve sample saturation. In RNA-Seq technologies, saturation could be reached when an increment in the number of reads does not result in additional true

expressed transcripts being detected or in more features called as differentially expressed when two or more conditions are compared [82]. However, the elevated costs usually associated with NGS-sequencing technologies make further experiment validation a more attractive option for researchers. The validation experiments consist of taking the same RNA samples initially used for cDNA library construction and re-analyzing them using a complementary technique, usually microarrays (for those species who already have this platforms available) [83,84] or RT-qPCR (quantitative reverse transcription PCR) [85].

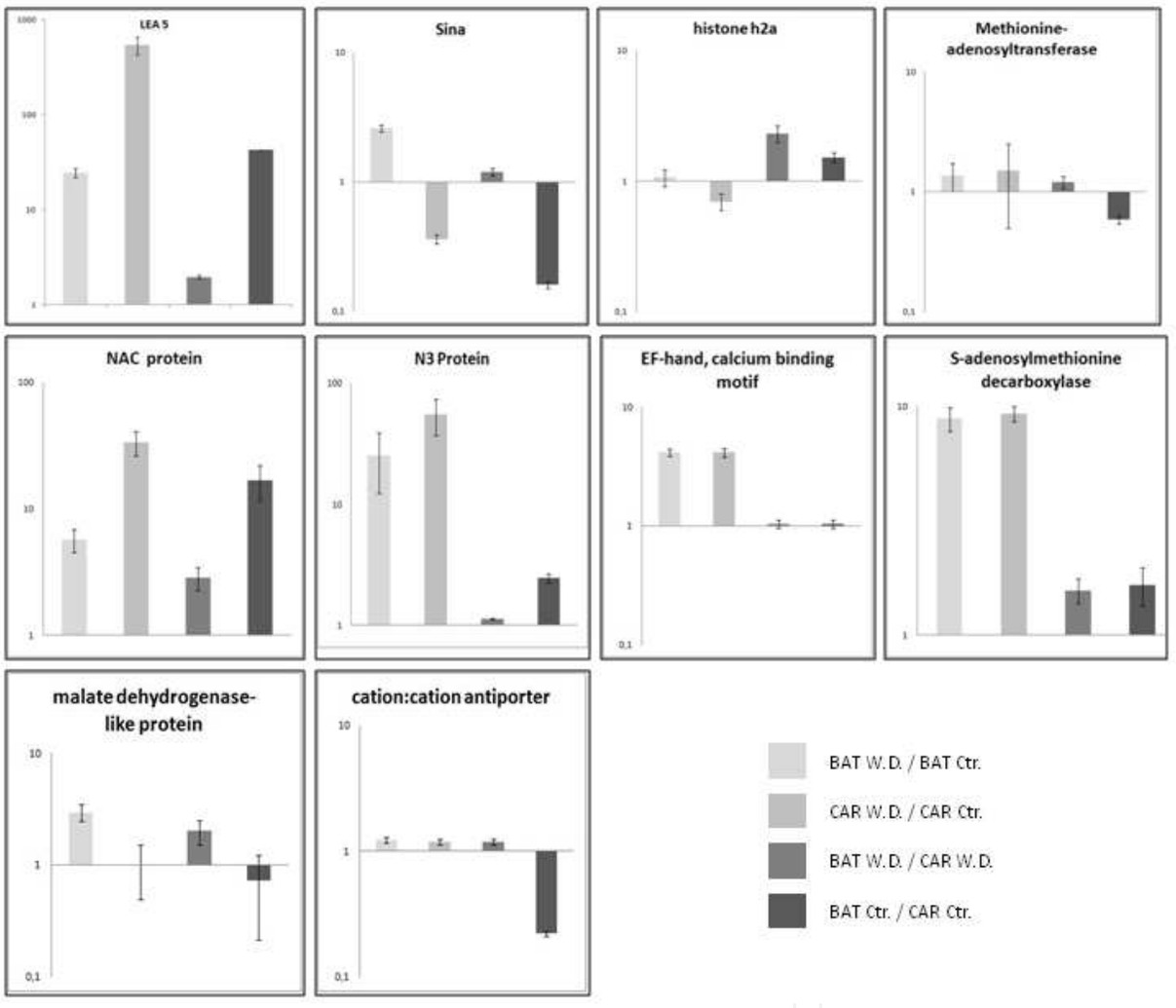

Figure 4. Relative gene expression profile of 10 ESTs selected from the BAT 477 drought stress-related SSH library [77]. Captions: BAT – BAT 477 drought-tolerant common bean genotype; CAR – Carioca 80 SH drought-susceptible common bean genotype; W.D. – 192h of water-deficit treatment; ctr. – control plants. BATWD/BATctr (BAT 477 under stress compared with BAT 477 control plants); CARWD/CARctr (Carioca 80SH under stress compared with Carioca 80SH control plants); BATWD/CARWD (BAT 477 under stress compared with Carioca 80SH under stress – SSH library initial condition); and BATctr/CARctr (BAT 477 control plants compared with Carioca 80SH control plants). (Extracted from [77]).

For the BAT 477 drought stress SSH library, it was selected as a set of 10 ESTs among those with most abundant contigs: *LEA5, Sina, histone h2a, methionine adenosyltransferase, NAC protein,*

N3 protein, EF-hand – calcium binding motif, S-adenosylmethionine decarboxylase, malate dehydrogenase-like protein, cation:cation antiporter. For each of the ESTs, a specific pair of primers for RT-qPCR analysis was designed [77] and gene relative expression quantification was obtained for the same tester and driver samples used for the SSH library construction (Figure 4). These results served well for the SSH library validation since all the selected transcripts revealed to be upregulated in BAT 477 plants under drought stress. Besides, for some of the transcripts (*LEA5, NAC protein, N3 protein, Ef-hand – calcium binding motif, and S-adenosylmethionine decarboxylase*), although they are expressed in lower concentrations on Carioca 80SH 192h drought-stressed plants, when compared to Carioca 80 SH controls, they undergo an even greater upregulation in relation to BAT 477 (Figure 4). This not only confirms the relevance of these transcripts on drought stress response regulation in common beans but also reveals that the drought-tolerant genotype BAT 477 may already keep a basal level expression of some important drought-related transcripts, thus stress perception by this drought-tolerant genotype may trigger more efficient signaling mechanisms that leads to a more discreet gene expression upregulation allowing the plant not to dislocate resources that otherwise may be saved for keeping homeostasis and therefore secure development and growth during the stress period.

4.2. DREB transcription factors as candidates for drought-tolerance improvement

Finding candidate genes and investigating their functional role and association with drought-tolerance traits and mechanisms have been of prime interest for many crop plants such as common bean. The DREB transcription factors subfamily has been studied in depth as candidate genes for breeding of abiotic stress tolerance. This group comprises a series of genes intermediating the regulation process to cope with abiotic stresses effects such as drought. They were originally described by [86], which identified a *cis*-acting regulatory element, DRE (dehydration responsive element), present in the gene promoter COR78/RD29A and involved in the response to drought, high salinity, and low temperature, further named as DREB (DRE-Binding). These proteins are capable of binding to DRE to activate the expression of genes of the stress signaling pathway. DREB transcription factors are unique to plant species and so far several genes have been described in *Arabidopsis* and other plants [87, 88].

The primary feature of a DREB transcription factor is the presence of a highly conserved protein domain, the EREBP/AP2. It was discovered within *APETALA2*, which plays an important role in flowering and seed development in *Arabidopsis.* Several proteins have been found containing this domain along their amino acid chain, consisting of a repeated motif of approximately 60 amino acids [89–91]. All these proteins are comprised in the larger superfamily EREBP/AP2 divided into three families referred as AP2, ERF, and RAV, based on their sequence similarity and the number of EREBP/AP2 domains [92]. The ERF protein family contains only one EREBP/AP2 domain and is subdivided into two main subfamilies, CBF/DREB and ERF [91]. The amino acids 14 and 19 of the EREBP/AP2 domain distinguish DREBs (valine and glutamic acid, respectively) from ERF (alanine and aspartic acid, respectively) [91]. In addition, ERF genes are involved primarily in responses to biotic stresses such as pathogenesis while DREB genes have main role in abiotic stresses responses.

DREB genes can be divided into six subgroups (A-1 to A-6). This categorization was based on phylogenetic trees as well as particular features related to their induction. The two most studied groups have been A-1 and A-2. Genes *DREB1*/*CBF* belong to subgroup A-1 and have been characterized as induced by low temperature in *Arabidopsis* [93], but other studies revealed some inducibility under drought and salinity as well [91, 94]. *DREB2* genes are primarily involved in responses to osmotic stress (dehydration and salinity) [91, 95].

Most of DREB findings have been associated with *Arabidopsis*; however, many studies have been performed with other species as well, revealing several new orthologs and different inducibilities for each one of the six DREB subgroups. Some of these findings have been done with legumes such as *Medicago truncatula* and *Glycine max*, close relatives to common bean.

Few studies have been published so far for common bean DREB genes, and they were mostly related to polymorphic sites identification along gene sequences. Ref. [96] categorized two orthologs *DREB2A* and *DREB2B* and identified polymorphisms between some Mesoamerican and Andean genotypes. Further investigation of these genes has been done to identify polymorphism patterns across wild and domesticated common beans. An attempt for phenotypic associations with drought-tolerance traits has been performed as well, but no clear patterns were obtained [18].

The research team of University of São Paulo, Brazil, has been studying DREB genes in depth. A pre-categorization study of the *PvDREB* gene subfamily has been done [97], showing putative DREB representatives for the species. Several genes have been isolated and their expression profiles determined under several abiotic stresses, including drought. One particular gene showed strong induction under many abiotic treatments, such as drought, salinity, and cold [98]. Some genes have been selected for a deeper molecular basis understanding as well as for their functional role in improving drought tolerance as well as other abiotic stresses.

Some other studies have found DREB genes in whole transcriptome profiles, such as in one experiment contrasting the drought-tolerant cultivar Long 22-0579 and the sensitive Naihua, in which a RNA-seq analysis was performed for samples under drought and control conditions. DREB transcription factors were identified to be differentially expressed and RT-qPCR analyses showed one transcript had the relative number of transcripts increased during the drought period [99]. Moreover, not only drought treatments have been analyzed but also one transcriptome profile has been done for a salt-tolerant bean cultivar named Ispir. It revealed several AP2/EREBP genes differentially expressed when contrasting a saline hydroponic solution with control conditions. Nevertheless, authors have not performed further categorization to identify which of those genes fitted *PvDREB*-specific characteristics [100].

Much more has to be done with DREB genes in common bean. Isolating and characterizing DREB genes for the species seems to be an important step toward the improvement of beans for abiotic stresses tolerance, especially for drought.

4.3. Phenotyping for drought tolerance in common bean

The identification of genomic regions or candidate genes, their functional role, and association with drought tolerance in common bean are fundamental aspects to understand the molecular signatures involved in acquiring such tolerance. However, in that purpose phenotyping methods are essential to effectively proving the effect of those genes on traits of interest. Thereby, it is important establishing and standardizing a phenotyping methodology to compare and select genotypes with different levels of stress tolerance in the studies one might be conducting. Furthermore, bringing data from the lab and greenhouse to the field is a big challenge, but of great importance for successfully applying the knowledge obtained about the genes, genotypes, and phenotypes of interest.

Phenotyping techniques have been developed to differentiating common bean accessions and cultivars for their levels of drought tolerance. Greenhouse trials have been applied to phenotype several shoot and roots traits and a common method employed has been the soil tube screening system assay that has been developed at CIAT [101]. Ref. [102] points out several traits that might be measured through such system, including many photosynthetic traits (photosynthetic efficiency, total chlorophyll content – SPAD, stomatal conductance, transpiration rates, leaf temperatures, leaf water potential), shoot and root biomass at the time of harvesting, leaf area and root traits (length, diameter, specific root length, and dry weight). Determination of root length might be done by image analysis system (WinRHIZO, Regent Instruments Inc.) [102] or might be manually determined by following root development on a graded plastic transparent tube in which plants were grown, all placed in PVC tubes.

The tube system developed by [101] was used to evaluate the effect of drought stress on root growth and distribution and compare different genotypes. Due to the difficulties of phenotyping roots in the field, this method has been shown to be a good complementary strategy applied in greenhouse conditions [102]. Examples in this sense are the studies of [103, 104] that analyzed the rooting patterns in greenhouse conditions with PVC soil cylinders and photosynthetic and yielding traits in different field areas. A population of recombinant inbred lines (RIL) from the crossing between the deep-rooting genotype BAT 477 and the small red-seeded and drought-susceptible DOR 364 was evaluated in both conditions. The greenhouse experiment showed that BAT 477 had significant larger root system based on root volume and deeper rooting ability, larger and thicker root, wide root diameter and biomass, under well-watered and progressive drought stress treatments [103].

For experiments conducted at the field, several traits can be evaluated since initial plant growth still harvesting. Ref. [102] made a very elaborated list with many parameters such as plant biomass at mid-pod filling and at harvesting time, seed yield, harvest index (HI), pod harvest index (PHI), drought intensity index (DII), and drought susceptibility index (DSI). The latter is based on the mean yields of a given genotype in drought stress and under no stress [102]. It assumes that one genotype will be more drought tolerant if the yielding is not so much reduced by the stress treatment in comparison to other genotypes. Pod harvest index has also been shown as a good indicator of drought tolerance, as shown by a field study in Ethiopia with the population from the crossing SXB 405 (breeding line) × ICA-Bunsi (white pea bean). Sensitive

lines presented significant reduction on PHI while no differences were observed for the most resistant lines [105].

Despite the availability of traits that might be evaluated in field conditions, the environment turns out to be a critical component interfering with results from one site to another. Drought field trials performed with the RIL population of the crossing BAT 477 × DOR 364, previously referred to the greenhouse experiment, showed significant variability across four locations evaluated [104]. A QTL analysis associating the field traits to a previous set of molecular markers disposed in a linkage map [106] showed significant QTL–environment interactions. Therefore, determining if one cultivar is tolerant to drought does not necessarily mean it will respond well to all environments, in a sense that it must be tested in multiple environments to check for its performance.

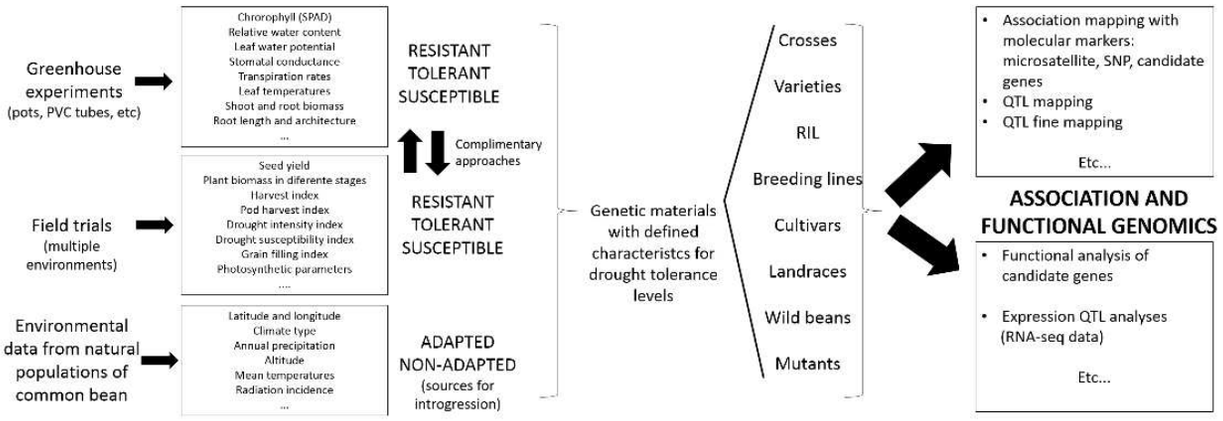

Figure 5. Strategies for phenotyping common beans for drought tolerance. Greenhouse experiments and field trials are complimentary strategies for evaluating several parameters and defining drought-tolerance levels. Recurring to wild beans based on environmental data might also be useful to select for beans adapted to drought episodes. Those evaluations altogether may be used toward the development of new varieties and the identification of genomic regions associated with the phenotypes evaluated as well as the functional role of candidate genes that are under characterization.

Although greenhouse and field methods have been developed to identify drought-tolerant genotypes and gene markers associated to such parameters, recent efforts have also been focused on the identification of sources of drought tolerance in wild beans spanning the natural area of distribution of *P. vulgaris* [107]. However, reliable estimations of drought tolerance in wild beans are not easy to establish, and attempts toward the development of new methods have been in course. Potential evapotranspiration models coupled with precipitation regimes were used to define a drought index for a series of wild bean accessions. Considering this factor along with the population structure might be a useful tool to analyze the levels of drought tolerance and use these materials for introgression of alleles of interest [107].

All these methods might be useful to carefully understand the phenotypic basis of drought tolerance variation in common bean genotypes. With standardized methods for the traits one might be interested, the accuracy between the association of molecular data and phenotypes might be much higher. It may be applied to QTL and association mapping studies, which link genome-wide molecular markers such as microsatellites, SNP, and gene-specific markers to

drought-related traits (103, 104, 106, 108]. On the other hand, standard greenhouse parameters can be used to test transgenic lines for determined candidate genes to verify their performances under imposed drought stress. Figure 5 shows a scheme of how greenhouse, field, and wild environment phenotyping studies might be useful for association and functional genomic studies in common bean.

5. Perspectives on the functional genomics of common bean

As mentioned before, common bean is not a species amenable for genetic transformation with the aim to test genes and to do functional studies. Thus, genomic mapping, transcriptomic and proteomic studies in contrasting genotypes, phases of development, different treatment/growth conditions, etc. are currently the most used approaches to identify genes linked to determined loci, verify changes in plant metabolism, and ultimately identify candidate genes suitable for molecular breeding or functional analyses.

The "omics" technologies and bioinformatics tools for large-scale data analysis have become essential to understanding the molecular systems that underlie various plant functions [109]. Despite common bean has been receiving increasing edible and economic importance, an investigation at a comprehensive omics level has been lacking in comparison to other model legume crops. As the genome sequences of *P. vulgaris* has become recently available, a new chapter has been opened for research with this crop. The genome release has provided a great miscellany of candidate genes that should be useful to improve common bean toward several different goals and approaches.

When considering abiotic stresses, some interesting NGS-related transcriptome data associated to drought [99] and salt-stress tolerance [100] as well as proteomic data related to drought [110], chilling [111], and osmotic stresses [112] have already been accessed. The consequential integration of a wide spectrum of omics data sets is then essential to promote translational research to engineer plant systems in response to the emerging demands of humanity.

Nevertheless, there is a big lack of information regarding interaction among stress sources. A recent trend for other crops has been the study of the effects of combined stress treatments such as drought versus salt, drought versus heating, drought × salt × nutrition, among others. These new studies try to represent most appropriately what really happens in the field, since plants are often subjected to multiple stresses. This should also be extended to the level of abiotic versus biotic stresses since many diseases are coupled with abiotic stresses at a certain stage of development of common bean. The available research on genomic, transcriptomic, and proteomic level on isolate stress-inductive factors should now be reunited in an attempt to elucidate the most complex phenomena involved in stress interactions. And, that should be extended to another level of complexity, which is establishing the interaction of both abiotic and biotic stress sources on common bean.

Regarding plant/pathogen interaction, until the moment the pathosystem *Phaseolus vulgaris*/*Colletotrichum lindemuthianum* was only investigated in an incompatible interaction. However,

there are other combinations of genotype and pathogen races that lead to a compatible interaction and remain to be studied in order to compare these systems and understand which mechanisms are really responsible for the resistance.

Still, considering plant/pathogen interaction, in the past years, the LMD (laser micro-dissection) technology has been applied to study individual cells of plant-infected tissue and/or pathogen structures. This is because the way plant tissues were collected to do quantitative analyzes, as transcriptomic and proteomic, could generate a dilution of those cells in direct contact with the fungus into the whole tissue. This type of analysis allows a specific and localized evaluation. The LMD technique is based on the coordinated use of microscopy, laser and robotic, to localize, dissect, and capture cellular material [113]. This method has been important in selection and sampling of cells or cellular content in enough quantity and quality for DNA, RNA, protein, and metabolite analyzes, even in high throughput. Our group is employing this technology to study *P. vulgaris*/*C. lindemuthianum* interaction and *P. vulgaris*/ mycorrhiza interaction under drought stress.

Looking for stress-resistance sources in other species and introgressing genes to common bean is another alternative for genomic improvement. A good example relies on the research that has been done for drought tolerance in common bean, based on interspecific crosses with other species of *Phaseolus*, such as tepary beans (*P. acutifolius*). They naturally span from the desert highlands of northwest Mexico to the southwest of the USA and thus they are good sources of drought, heat, and cold tolerance [114]. An interesting feature of tepary beans is their root system, which reveals extremely fine roots with rapid penetration in the soil with profuse branching, which enables quick access to limited soil water [115].

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