COMPARATIVE TRANSCRIPTOME PROFILING OF BACTERIA-MEDIATED DROUGHT TOLERANCE IN MAIZE (*ZEA MAYS* L.) SEEDLINGS.

A Thesis

by

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ABSTRACT

Drought is considered the most important limiting factor in crop production all around the world and it negatively impacts plant growth and productivity. Many studies have suggested that plants recruit specific microbiomes depending on their environmental conditions and some bacteria have been shown to be able to alleviate drought stress in plants. The present research project aims to allow a better understanding of the molecular mechanisms underlying the ability of these bacteria to confer drought tolerance to plants by performing whole transcriptomic analysis using RNA sequencing. The mRNA transcriptomes from maize (*Zea mays* L.) seedlings were studied 5 and 9 days after inoculation with 2 different bacteria, *Bacillus* sp. 12D6 and an *Enterobacter* sp.16i known to confer drought resistance to maize. The data obtained were aligned to the maize genome using the STAR aligner and the reads were counted using Htseq and the differential gene expression analysis was performed using EdgeR. The gene ontology analysis was performed using ShinyGO and the KEGG pathway analysis with KEGG mapper. Our results show different and common pattern for the regulation of genes associated with Ethylene biosynthesis, Abscisic acid, Auxin signaling, Superoxide dismutase, Catalase, Peroxidase, Heat shock proteins, and late embryogenesis abundant protein in the seedlings inoculated with the two bacteria. These results have provided useful knowledge on the beneficial actions of these two bacteria.

DEDICATION

I dedicate this thesis to my wife, Ziara, my children, Abdoul-Rahmanou, Zakhiya, Hamida, and my parents, Zalia and Idrissou.

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NOMENCLATURE

ABA:	Abscisic Acid
ACC:	1-Aminocyclopropane-1- carboxylate
DEG:	Differentially Expressed Genes
dpi:	Days post-inoculation
GO:	Gene Ontology
HSP:	Heat Shock Protein
IAA:	Indole-3-Acetic Acid
JA:	Jasmonic acid
JAZ:	Jasmonate ZIM domain
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LEA:	Late Embryogenesis Abundant
mRNA:	messenger Ribonucleic Acid
OD:	Optical Density
PBS:	Phosphate-buffered saline
PGPR:	Plant Growth-Promoting Rhizobacteria
RNA:	Ribonucleic Acid
RNA-Seq:	RNA Sequencing
ROS:	Reactive Oxygen Species
RWC:	Relative Water Content
SA:	Salicylic Acid
SnRKs:	SNF1-related protein kinase 2 s

SPS:	Sucrose phosphate synthase
STAR:	Spliced Transcripts Alignment to a Reference
TF:	Transcription Factors
TP:	Time Point
TPP:	Trehalose-6-phosphate phosphatases
W_Neg:	Water Negative (Absolute negative control)
W_Pos:	Water Positive (Absolute positive control)
ZIM	Zinc-finger inflorescence meristem

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

As a major abiotic stress factor, drought affects crop and pasture yield in arid and semi-arid areas (Odokonyero et al., 2016). This stress negatively affects plant growth and productivity worldwide in a period when an increase of agricultural production is needed to feed the growing human population (Anjum et al., 2011, Xu et al., 2018, Hubbard et al., 2014). According to Lesk et al. (2016), during the last decades, drought was responsible for an average 13.7% loss in cereal production worldwide.

Plants, because of being sessile, have developed appropriate adaptation strategies to ensure their survival and reproductive success (Barnabás et al., 2008). One of these strategies is the formation of mutualistic relationships with microorganisms living in their surroundings. Plants provide specific habitats for these beneficial microbes living in their rhizosphere, phyllosphere, and endosphere (Berg et al., 2015). The plant-microbe interactions in the rhizosphere have been actively studied due to their huge potential for plant health promotion (Hirsch & Mauchline, 2012). Some of the beneficial interactions have been associated with germination stimulation, host physiological development, and resistance to biotic and abiotic stresses (Santos-Medellín et al., 2017, Berg et al., 2015, Orozco-Mosqueda et al., 2018, Oyserman et al., 2018). Our understanding of the intimate co-dependent relationship between plants and their microbiome has led to the concept of plant growth-promoting rhizobacteria (PGPR). PGPR are bacteria that live in the root endosphere and the rhizosphere soil surrounding plant roots which provide the plants with

diverse benefits such as increased availability of nutrients, the control of plant pathogenic microbes, and tolerance to abiotic stresses, including drought (Skz et al., 2018).

At the molecular level, PGPR conferring drought tolerance to plants use several mechanisms such as (i) production of phytohormones like indole-3-acetic acid (IAA), cytokinin and abscisic acid (ABA); (ii) production of bacterial exopolysaccharides having the ability to improve drought tolerance in certain plants; (iii) production of Bacterial 1-aminocyclopropane-1- carboxylate (ACC) deaminase which hydrolyzes ACC into ammonia and alpha-ketobutyrate, with ACC acting as an immediate precursor of ethylene during stresses; (iv) induction of systemic tolerance to biotic and abiotic stress; and, (v) promotion of plant growth and development (Kumar & Verma, 2018, Farooq et al., 2009). Much of the PGPR related research has focused on exploring the wide range of growth-promoting species in the arsenals of these microorganisms. However, the mechanistic understanding of the host plant's molecular response is seldom explored. This information is vital in evaluating the success of an introduction of PGPR in the field.

Transcriptomics through next generation sequencing allows untargeted exploration of host plant genetic responses to PGPR. Several studies have used this method to elucidate the biochemical and physiological mechanisms underlining the beneficial effects of PGPR to host plants. There have been distinct patterns in terms of transcript abundance in PGPR-inoculated plants, compared with non-inoculated controls. In the study by Hardoim et al. (2019), where the authors inoculated maize plants with two PGPR, *Azospirillum brasilense,* and *Herbaspirillum seropedicae,* the results showed that there were identical and unique patterns in terms of the plant transcriptome induced by the

two microbes. In another transcriptomic study by Skz et al. (2018), maize seedlings inoculated with a *Pseudomonas putida* strain were shown to downregulate the expression of genes related to ethylene biosynthesis, abscisic acid (ABA) and auxin signaling, superoxide dismutase, catalase, and peroxidase. However, upregulation was observed with genes associated with β-alanine and choline biosynthesis, heat shock proteins, and late embryogenesis abundant proteins. The regulation of these genes is linked to better tolerance of plants to drought. To sum up, each PGPR has its mode of action in helping plants cope with biotic or abiotic stresses and these modes of action are linked to the regulation of specific genes in the plants. The present project aims to study the mode of action of two different PGPR on maize and to detect genes linked to their beneficial action.

The alleviation of drought stress in plants is linked to several mechanisms of action and the understanding of these variations is a key that will allow the application of microbiome-based solutions against drought. To be successful, the introduction of a PGPR strain has to be compatible with (i) the host plant (ii) climatic and seasonal conditions (iii) soil conditions (iv) the other microbes present in the soil at the time of application. As an endeavor towards this greater goal, the present study will focus on studying the variation of host response after inoculation with two different PGPR.

Two PGPR isolates, *Bacillus* species, 12D6, and *Enterobacter* species, 16i, were isolated in the Jo Lab by Jochum et al. (2019), for their ability to confer drought tolerance to maize and wheat. These two PGPR strains from two different species showed promising drought resistant responses when the rhizospheres of maize and wheat seedlings were inoculated with these two beneficial bacteria resulting in a delayed onset of plant drought

symptoms. In addition, their presence in the rhizosphere was associated with a significant increase in root length, root surface area, and number of root tips when compared to the uninoculated control. The phytohormone profiling of the two beneficial bacteria grown *in vitro* showed that both strains produced indole-3-acetic acid (IAA) and salicylic acid in relatively high amounts. Following this study, the next important step is the understanding of the mode of action of these beneficial PGPR during their interaction with plants under drought stress. Belonging to two different species, this understanding will provide an insight into the overall beneficial action of PGPR on plants under drought.

The objective of the present project, therefore, is to perform a whole transcriptome analysis of maize seedlings under drought stress after inoculation with the two bacteria, *Bacillus* 12D6 and *Enterobacter* 16i. The present study hypothesizes that there are similarities and differences in terms of genes expressed by maize seedlings under drought after inoculation with the two PGPR.

Literature review

Drought constraint in agriculture

Drought is defined as a prolonged shortage in the water supply to plant crops and it accounts for nearly 70% potential yield losses worldwide (Fracasso et al., 2016, Odokonyero et al., 2016, Zenda et al., 2019) and is considered the most critical abiotic stress in terms of its impact on crop productivity reduction, as well as plant growth and development. Drought severity and length are critical, and yield losses induced by this constraint can exceed losses from other constraints like diseases, insects, or weeds (Farooq et al., 2009, Zolla et al., 2013). During the last decades, drought was responsible for many

famines, and nowadays, with the dearth of water resources, drought is considered the most serious constraint to world food security and responsible for an average 13.7% loss in cereal production worldwide (Lesk et al., 2016).

On a global scale, statistics show that irrigated cropland only covers 20% and contributes only to 40% of worldwide food production, while the remaining 60% is provided by rain-fed agriculture (Ojuederie et al., 2019). This fact means that the majority of the crops feeding humanity can be subjected to drought stress and unfortunately in the next few years, as a consequence of an increase of evapotranspiration and decrease of precipitation due to global warming, droughts are expected to be more frequent and more severe. Sadly, world freshwater availability is limited and the expected boom of the world population will increase the competition for freshwater among agriculture and other uses of water because, from the current population of about 7 billion people, it is projected that the global population will probably increase to 10 billion or more in the next 50 years (Tombesi et al., 2018, Ojuederie et al., 2019).

In order to make agriculture more efficient and aid in the development of drought-resistant crops, it is important to understand how plants behave when confronted with water scarcity and which adaptation strategies are developed to cope with this stress. This understanding is important for the genetic improvement and breeding for tolerance to this stress (Zolla et al., 2013, Lv et al., 2018).

In order to mitigate the effect of drought on plant development and production, there are initiatives around the world to implement sustainable approaches by creating

drought-tolerant plants, shifting crop calendars, optimizing fertilizer use, using good agricultural practices, and resource management. (Espidkar et al., 2017).

The sessile characteristic of plants has allowed them to develop appropriate adaptation strategies through evolution in order to guarantee their survival and reproductive success (Barnabás et al., 2008). One of these adaptation strategies is to build mutualistic relationships with other organisms especially microbes living in their surroundings.

Plant growth-promoting rhizobacteria

Plants and their direct environment constitute habitat for diverse microorganisms including bacteria, fungi, nematodes, and algae (Ji et al., 2014). The important role of microbes living in the roots for plant growth and health was acknowledged as early as 100 years ago. These associated plant microbes interact with their host in essential functional contexts by stimulating germination and development, by helping plants resist or tolerate biotic and abiotic stresses, and by impacting plant fitness (Santos-Medellín et al., 2017, Berg et al., 2015, Orozco-Mosqueda et al., 2018, Oyserman et al., 2018). Plants then significantly rely on their associated microbial communities for nutrient uptake and protection against stresses and they are known to recruit a specific group of microbes depending on their environmental conditions (Santoyo et al., 2016, Bakker et al., 2018).

Plants provide specific habitats for their associated-microbes and these habitats can be categorized as the rhizosphere, the phyllosphere, and the endosphere. Among these three microbial community habitats, the rhizosphere is the most studied due to its huge potential for plant nutrition and health improvement. The rhizosphere is colonized by

different microbes present in and around the roots. These microbes have developed several types of interactions depending on the plant's nutrient status in soil, the soil characteristics, the plants' defense mechanisms, and the specific microorganisms present in the rhizosphere zone of influence (Hirsch & Mauchline, 2012, Parmar & Dufresne, 2011). This means that the plant-associated microbiome is not a passive player in this interaction and explains why plants are believed to actively recruit specific microbiomes when confronted with specific stresses (Berendsen et al., 2012).

PGPR are bacteria living in the rhizosphere surrounding plants and which provide plants with diverse benefits such as increased availability of nutrients, the control of plant pathogenic microbes, and tolerance to abiotic stresses, including drought (Skz et al., 2018). The manipulation of the plant microbiome (a specific group of microbes recruited by a plant) is becoming a reliable strategy to overcome the negative effect of drought on plants as reported by several authors (Jochum et al., 2019, Farooq et al., 2009, Kumar & Verma, 2018). Hence, the understanding of mechanisms used by these PGPRs to enhance plant health is important for the global uses of these beneficial microbes in agriculture.

Several physiological and molecular mechanisms have been proposed to explain the actions of PGPRs in drought stress tolerance mediation in plants. These PGPRs are reported to favor plant growth and development under water stress conditions using several mechanisms such as (i) production of phytohormones like IAA, cytokinins and ABA; (ii) production of bacterial exopolysaccharides that confer improved drought tolerance in certain plants; (iii) production of bacterial ACC deaminase which hydrolyzes ACC into ammonia and alpha-ketobutyrate; (iv) induction of systemic tolerance; and, (v)

promotion of plant growth and development (Kumar & Verma, 2018, Farooq et al., 2009). For instance, bacteria from the genera *Azospirillum*, known for the production of phytohormones, promote plant growth and induce drought tolerance in wheat. *Pseudomonas chlororaphis* O6, a bacterium producing 2R, 3R-butanediol, induces stomatal closure genes and reduces water loss in *Arabidopsis thaliana*. *Achromobacter piechaudii* ARV8 a bacterium producing 1-aminocyclopane-1-carboxylate -deaminase, lowers ethylene levels and induces drought tolerance in tomato and pepper seedlings. *P. putida* strain GAP-P45 was reported to enhance plant biomass, relative water content, and leaf water potential by the accumulation of proline in maize seedlings exposed to drought stress. *Bacillus* sp. enhance drought tolerance in maize seedlings by the reduction of antioxidant enzyme activity such as ascorbate peroxidase and glutathione peroxidase (Skz et al., 2018).

Transcriptome profiling and RNA-seq

For a specific physiological condition, the transcriptome is referred to as, the whole set of transcripts present in a cell, and their quantity (Wang et al., 2009). The most important aim of profiling a transcriptome is to characterize and quantify the changing expression levels of each transcript during development and under different conditions in order to define cellular state at a specific time or to identify genes with similar expression patterns. The most recent and most effective method of transcriptome profiling is RNA sequencing or RNA-seq using deep-sequencing technologies. (Wang et al., 2009, Rani & Sharma, 2017).

Transcriptome profiling of plants under drought inoculated with PGPR

Plant transcriptome profiling has provided a tool for understanding the mechanisms by which plants respond to stress conditions and RNA-seq has been used to characterize changes in the transcriptomes of plants confronted by abiotic stress (Song et al., 2017). Plants usually respond to drought stress or inoculation with beneficial microbes by activating a complex gene regulatory network and multiple signaling pathways (Zhang et al., 2017). Several studies have used the transcriptome profiling of plants under drought inoculated with PGPR compared to non-inoculated controls to elucidate the biochemical and physiological mechanisms underlining tolerance to drought or the mechanisms behind the beneficial effects of these PGPR. These studies have allowed the characterization of many genes associated with different biological pathways.

*Genes associated with stress signal transduction***:** According to Frolov et al. (2017), the first step to ensure a plant's survival upon exposure to abiotic stress like drought, is the signal perception linked to this stress. Drought is first perceived by receptors present on cell membranes and transduced downstream resulting in the generation of secondary messengers including K^+, Ca^{2+}, sugars, ROS, cyclic nucleotides, and inositol phosphates. After perception by these receptors, the secondary messengers then adjust the intracellular calcium content. This disturbance of the cytosolic Ca^{2+}level is perceived by calcium-binding proteins known as Ca^{2+}sensors. These messengers eventually elicit the downstream signaling pathways which will lead to the alleviation of the stress by the alteration of gene expression (Zheng et al., 2020).

Genes related to carbohydrate metabolism: The catabolism of carbohydrates is very important for plant survival by providing important saccharides and energy required for cell function (Min et al., 2016). Zheng et al. (2020) after conducting work similar to the one conducted by Zenda et al. (2019) reported 37 genes in tolerant line associated with starch synthesis, such as granule-bound starch synthase and beta-amylase, which were upregulated contributing despite drought to augment carbohydrates reserves in the plant. A transcriptome profiling conducted on maize seedlings inoculated with a beneficial bacteria (*P. putida*) by Skz et al. (2018) showed that some genes involved in starch synthesis were downregulated and several genes involved in the starch break down, like β-amylase isoforms, were downregulated while α-amylase were upregulated. The genes associated with sucrose synthase 7 and sucrose phosphate phosphatase were upregulated. Two other studies on plants inoculated with beneficial microbes under drought show that the beneficial action of *Bacillus subtilis* on Timothy grass and *Neotyphodium coenophialum* in Tall fescue plants were linked to the accumulation of sucrose (Nagabhyru et al., 2013, Gagné-Bourque et al., 2016).

Genes related to membrane transporters: Aquaporins, present in the plasma of plant cells, are integral membrane channel proteins, that facilitate the transport of water and/or small neutral solutes or gases (Maurel et al., 2008). Plant aquaporins are part of the gene family of the major intrinsic proteins (MIPs). Based on amino acid sequence similarity, this gene family is subdivided into five subfamilies, the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin-26-like intrinsic proteins (NIPs), the small basic intrinsic proteins (SIPs), and the uncharacterized

intrinsic proteins (XIPs). Each subfamily is divided into groups. Among these subfamilies, the PIPs are the main regulators of plant water absorption (Skz et al., 2018). The results of transcriptome profiling on maize seedlings inoculated with *P. putida*, by Skz et al. (2018), and arbuscular mycorrhizal fungi (AMF) by Quiroga et al. (2017), Armada et al. (2015) and Bárzana et al. (2014) show downregulation of ZmPIP 1; 3 encoding gene. Quiroga et al. (2017) reported the upregulation of two genes encoding ZmPIP2; 2 and ZmPIP2; 4 in AMF inoculated maize drought-tolerant cultivars. Skz et al. (2018) showed that the 4 PIP2; gene was upregulated, while the PIP1; 5, PIP2; 1, PIP2; 2, and PIP2; 6 genes were downregulated. ZmTIP1; 1, ZmTIP2; 3, and NIP2 encoding-genes were downregulated and TIP4;1 was upregulated (Quiroga et al., 2017). NIP, NIP1; 1, and TIP4; 2 were downregulated (Skz et al., 2018). These previous studies suggest that the downregulation of aquaporins reduces water flow through cell membranes and maintains leaf turgor thereby helping seedlings from being affected by drought stress compared to the uninoculated seedlings.

*Genes related to the Reactive oxygen species (ROS) scavenging system***:** In response to drought stress, plants produce and accumulate, in a rapid and transient manner ROS of which when accumulating beyond safe levels is known to damage cellular components and structures (Ahmadi et al., 2010). ROS interact with proteins, lipids, and deoxyribonucleic acid, leading to oxidative damage that impedes the normal functions of the plant. In order to avoid the toxic effects linked to excessive accumulation of ROS, plants have evolved detoxification mechanisms including antioxidant molecules and enzymes that can act to avoid the toxic effects. To surmount the oxidative stress, plants

use antioxidant defense systems constituted by enzymatic and non-enzymatic antioxidants which inhibit the accumulation of ROS and reduce the oxidative damage (Vurukonda et al., 2016). The enzymatic antioxidants are constituted by ascorbate peroxidase, catalase, glutathione peroxidase, superoxide dismutase, and peroxiredoxin and the non-enzymatic components contain ascorbate and glutathione. To restore the cellular redox balance and homeostasis in response to drought, plants modify their metabolism by different means. This modification can be done by the production of osmoprotectants, like proline, which reorganizes proteins and cellular components, and maintain cell turgor by osmotic adjustment and modifying the antioxidant system (Mahajan & Tuteja, 2005, Gong et al., 2005). Genes triggered by drought, such as the LEA proteins, dehydrins, heat shock proteins (HSPs), and other molecular chaperons are adjusted in response to stress.

Genes related to hormone metabolism: Physiological and biochemical responses of plants to drought involve alterations in the metabolism of endogenous hormones, such as ABA, auxin, cytokinin, salicylic acid, and ethylene. ABA is the main stress response-related endogenous hormone in plants and when its content increases in plants under drought stress conditions, ABA induces stomatal closure, controls water vapor transpiration, and induces the expression of drought-resistance genes and the increased abundance of stress proteins. It should be noted that various interactions coordinate the regulation of plant endogenous hormones. In order to regulate the opening and the closure of stomates in plants facing drought-stress, the cytokinin content is significantly decreased, while the ABA content is significantly increases, thus activating the stress response. Cytokinin interacts with ABA that helps plants overcome water deficits by

activating a complex series of events leading to stomatal closure. This means that maintaining the perfect balance between the cytokinin and ABA contents can be surmised as an important mechanism in maize for surviving drought stress. An important hormone in response to environmental stresses is Auxin, which also interacts with ABA in the regulation of root growth and seed germination. Auxin and ABA are also antagonistic in the stomatal closure process. Another important hormone in drought stress response is ethylene. The compound 1-Aminocyclopropane-1-carboxylic acid, a precursor of ethylene, is transported from roots to shoots through the xylem increasing ethylene content in leaves under drought-stress. An Increase in ABA content leads to the inhibition of ethylene synthesis; which in turn promotes root elongation and an increase of water absorption by plants under drought-stress conditions. When the biosynthesis of ethylene is decreased grain yield of maize is improved under abiotic stress conditions whereas an increase inhibits plant growth (Zheng et al., 2020). In the ABA signal transduction pathways, there are three protein classes: (i) the pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor (*PYR/PYL/RCARs*) which are suggested to be the ABA receptors; (ii) *protein phosphatase 2Cs (PP2Cs)* acting as negative regulators of ABA production, and (iii) SNF1-related protein kinase 2s (*SnRKs*) acting as positive regulators. In the study by Skz et al. (2018), important ABA regulators such as PYL3, and PP2C isoforms such as PP2C, 2C 50, 2C ABI1, 37, and 68 were downregulated. The results also showed that the *SnRK2* family proteins such as plant-specific Serine/Threonine kinases were upregulated. In another study involving sugarcane transcriptome profiling following inoculation with *Gluconacetobacter diazotrophicus*, a

beneficial bacteria that also confers drought resistance on their host plant, showed that PYL8, PP2C, and SnRK2 were downregulated (Vargas et al., 2014).

According to Grover et al. (2011) in the ethylene biosynthesis pathway, S-adenosylmethionine (S-AdoMet) is converted by 1-aminocyclopropane-1-carboxylate synthase into the immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylate. When plants are under both biotic and abiotic constraints, ethylene endogenously regulates the homeostasis of plants and it results in the reduction of growth. Bacteria reduce the detrimental effect of ethylene, mitigating plant stress, and promoting plant growth (Glick, 2005, Vurukonda et al., 2016). In the studies by Skz et al. (2018) and Vargas et al. (2014), inoculation with beneficial microorganisms resulted in the downregulation of ethylene biosynthesis genes *ACO35* and *ACS47*.

Auxin is a hormone that plays a crucial role in all aspects of plant growth and development. The most studied auxin signaling gene families are *SAURs*, *GH3s*, and *Aux/IAAs*. In wheat, auxin is known as a negative regulator of drought tolerance, and this stress tolerance was associated with a decrease in IAA content in the plant (Vargas et al., 2014). The studies by Skz et al. (2018) and Vargas et al. (2014) reported that inoculation with beneficial microbes resulted in the downregulation of genes encoding GH3, GH3.8, SAUR56, and AUX/IAA.

Downregulation of IAA was reported to promote the accumulation of LEA mRNA, leading to drought stress adaptation in plants (Zhang et al., 2009). In the study by Skz et al. (2018), the bacterial inoculation led to an upregulation of genes encoding LEA proteins. The authors suggested that the downregulation of auxin signaling and the accumulation of

LEA proteins were related to drought tolerance in maize seedlings inoculated with *P. putida*.

Genes related to transcription factors: According to Shinozaki et al. (2003), in response to drought, transcription factors (TFs) are important in signal transduction in plants. TFs are encoded by almost 7% of the coding sequence genomes, and some of the genes related to them respond to short-term stresses. The simultaneous expression of lots of downstream stress-related genes can control the overexpression of stress response-related transcription factor genes (Zheng et al., 2020). The transcription factors have been labeled as the most important regulators of abiotic stresses, including drought, by acting on many downstream stress-responsive genes and many transcription factor families like MYB/MYC, WRKY, bZIP, DREB (AP2/ERF) and NAC which were well-known to be linked to drought stress (Chen & Zhu, 2004, Wang et al., 2016). The WRKY70 transcription factor is linked to disease resistance and is indispensable in the jasmonic acid/salicylic acid signaling pathway (Li et al., 2004). Another important transcription factor, ERF018, is associated with the development of plants' vascular bundles and is essential for their primary and secondary growth. This transcription factor is also associated with the ethylene pathway; the increased expression promotes cell division and vascular bundle elongation.

Importance of root measurement in drought phenotyping

According to Comas et al. (2013), there is a positive correlation between plants with greater linear root length, greater root surface area, and abundance of root tips and better tolerance to water scarcity and an overall increase in the maintenance plant

productivity. This fact explains the importance of studying plant's root system architecture under drought. A plant's water absorption ability is linked not only to its root system length and surface area, which contribute to better soil exploration but also to the proliferation of higher-order roots resulting in more root tips (Ngumbi & Kloepper, 2016, Barnawal et al., 2017). In previous studies, reductions in a the plant's root diameter were considered beneficial by enabling them to grow faster and to rapidly, acquire soil resources through root system extension coupled with a lower investment in dry biomass (Birouste et al., 2014, Walh & Ryser, 2000).

Leaf relative water content in drought phenotyping

In terms of the physiological consequence of cellular water deficit, the relative water content (RWC) is seemingly the most pertinent measure of plant water status. The relative water content technique is accepted worldwide as a reproducible and valid index of plant water status. The water status is a critical factor allowing the measurement of water stress and drought tolerance in lots of plants. The most commonly used plant tissues for RWC determination are leaf tissues. In drought situations, the leaf RWC provides an understanding of the mechanism of water utilization for the maintenance of cellular hydration (Smart & Bingham, 1974, Odokonyero et al., 2016).

Rationale of the present work

The alleviation of drought stress in plants is linked to several mechanism of action and the understanding of these variations is a key that will allow the application of microbiome-based solution against drought. In order to be successful, the introduction of

a PGPR strain has to be compatible with (i) the host plant (ii) climatic and seasonal conditions (iii) soil conditions (iv) the other microbes present in the soil at the time of application. As an endeavor towards this greater goal, the present study will focus on studying the variation of host response after inoculation with two different PGPR.

To date, in the area of transcriptome profiling of plant inoculated with beneficial bacteria, a lot has been done in order to decipher the molecular pathways involved in the beneficial effects of microorganisms-colonizing plants. However, less work has been done to understand the molecular mechanism underlining the beneficial action of PGPR conferring drought alleviation to plant. In addition, less is done to understand the changes in plant transcriptome over time when they are inoculated with PGPR under stress. The understanding of this mechanism will allow the identification of candidate genes that can possibly be used to improve drought resistance in maize. The present research project aims to provide a better understanding of the molecular mechanisms underlying the ability of two bacteria, *Bacillus* 12D6 and *Enterobacter* 16i, to confer drought tolerance to plants by performing a whole transcriptomic analysis over time on maize seedlings under drought inoculated with these bacteria. The study will be focused on the evolution of the transcriptome in time after inoculation with these beneficial bacteria in comparison with transcriptome of non-inoculated well-watered plants and none-watered plants.

CHAPTER II

MATERIAL AND METHOD

Experimental design

Inoculum preparation and Inoculation

For the inoculation, the two PGPR, *Bacillus* sp. 12D6 and *Enterobacter* sp. 16i, identified by Jochum et al. (2019) as able to confer drought tolerance to maize and wheat, were used. The bacterial inoculation method described by Jochum et al. (2019) was also used with slight modifications. At a glance, overnight cultures of the two PGPRs were grown in Luria-Broth (LB) at 30°C with gentle shaking (150 rpm). Bacterial cells were collected via centrifugation (5000 rpm for 2 min) and re-suspended in an equal volume of 0.1 M phosphate buffer saline (PBS composed of 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 18 mM KH_2PO_4).

Soil inoculum was added as aliquots of 200 µl of resuspended bacterial biomass applied to the soil at the base of each seedling 10 days after planting. Separate inoculation with 0.1 M PBS was used as a no-inoculum control. Inoculum densities were normalized to ensure populations of approximately 10^7 colony-forming unit (CFU) ml^{-1}.

Drought Tolerance Phenotyping prior to RNA seq experiment

Five- and nine-days post-inoculation, plants were examined for drought symptoms such as wilting, leaf curling, and marginal leaf necrosis, and visual scores were assigned to each of them. The scale used for visual score assessment is the one used in the Dr. Jo lab at Texas A&M University. It is composed of 5 levels:

- 5: no drought symptoms
- 4: slight wilt of the flag leaf
- 3: no lodging, moderate leaves drooping on both leaves
- 2: complete lodging, minor/moderate leaf curling, noticeable wilting
- 1: complete lodging severe leaf curling, noticeable stunting, permanent wilting.

Plant Materials and Growth Conditions

The seeds maize variety B73 were surface sterilized using Tween 20 for 1 hour followed by a subsequent multiple wash to eliminate all the Tween 20. Seeds were pre-germinated on sterile filter paper placed inside sterile Petri dishes at 25°C for 24h. There were between 50 and 55 seeds per dish of 8 cm of diameter and these seeds were planted separately in conical pots (4 cm diameter and 20 cm length) filled with sterilized germination mix (Jolly Gardener, PRO-LINE, C/GP Germination Mix, Atlanta, Georgia). The germination mix sterilization was done in an autoclave at 120ºC for 1 hour. The seedlings were watered with distilled water at field capacity for 10 days, inoculated with the two bacteria, and exposed to drought by withholding water. The beneficial effect of the two PGPRs was assessed by measuring the RWC and the root system architecture.

Determination of different time points for the experiment

In order to choose key time points, which will allow for an understanding of the bacterial-mediated drought tolerance, a mock test was conducted. Maize seeds were planted as described previously and water was withheld after 10 days. The plants were left under drought until 90% of the plants showed severe symptoms (complete lodging,

drooping, wilting). The results obtained showed that 11 days after water withholding (21 days after planting) 90% of the plants were showing severe drought symptoms. To avoid using very dry roots for the experiment, we decided to collect the final samples of the experiment at 9 days after drought (19 days after planting). An intermediate time point, 5 days after withdrawal of water (15 days after planting) was also chosen for evaluation as well. The tenth day of watering the plants (day 0 of withholding water) was chosen as the initial time-point for evaluation (Figure 1).

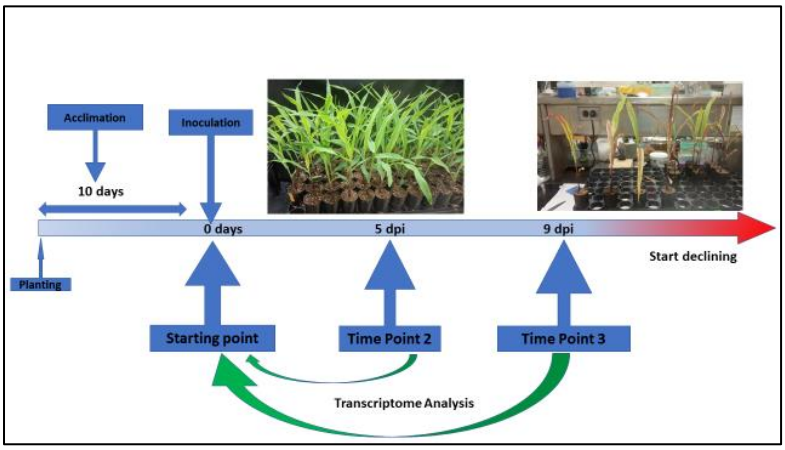

Figure 1: experiment summary

The seeds are planted and acclimated for 10 days and inoculated with the two bacteria. The inoculation serves as a starting point for the experiment. Two other time points were considered, 5 dpi and 9 dpi. After 9 dpi, the plants started decline and died. The transcriptome analysis will be done comparing the two last time points to the first one.

Root measurement

To assess the beneficial effects of the two PGPRs on maize root systems under drought stress, inoculated and non-inoculated plant roots were sampled 5 and 9 dpi. Maize seedlings roots were removed from the germination mix, with special care to preserve the

intact root system, the above part was cut off and roots were washed and stored in 4°C prior to measurement. Root architecture was measured by scanning the roots with a flatbed scanner (EPSON Perfection V800 Photo), calibrated for root measurement. Images obtained from the scanner were analyzed using WinRHIZO Pro 2019a generating total root length, root surface area, average root diameter, number of root tips, and root branching estimation (Himmelbauer et al., 2004)**.**

Leaves relative water content (RWC)

For RWC determination during our experiment, steps described by Teulat et al. (2003) were used. Briefly, the entire flag leaf of maize plants (leaf protecting newly produced leaf) was cut and the fresh weight (FW) was recorded immediately, followed by hydrating the sample overnight by immersing in water and turgid weight (TW) was recorded after blotting the leaf sample gently. The samples were dried at 60°C until constant dry weight (DW) was observed. RWC was calculated according to the formula:

$$RWC\ (\%) = [(FW-DW) / (TW-DW)] \times 100.$$

Transcriptome profiling

Sample collection and RNA extraction

Twenty-seven samples (see Table 1 for roots repartition per treatments) of maize seedlings roots collected for transcriptome profiling were removed from the germination mix with special care to preserve the intact root system, cut from the plant's above part, wrapped in aluminum foil and flash-frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction (Figure 2).

Figure 2: steps for roots collection for RNA seq.
Plants were taken out of the pots and the germination mix is clean off the roots. The roots are cut, wrapped in aluminum foil, put in liquid nitrogen and frozen in -80˚C.

Table 1: Number of samples collected for the RNA seq experiment

Treatments	1st time point (inoculation)	2nd time point (5 dpi)	3rd time point (9 dpi)
Control (baseline)	3 samples	-	-
12D6	-	3 samples	3 samples
16i	-	3 samples	3 samples
Well-watered plants (W_pos)	-	3 samples	3 samples
Plants under drought (W_neg)	-	3 samples	3 samples

The total RNA from roots was extracted using the RNeasy Plant Mini Kit (Qiagen, Massachusetts, USA) following the manufacturer's instructions. The quality of the total RNA was accessed using a **NanoDrop** and 1% agarose gel electrophoresis (Table 2).

Table 2: Quality results of RNA with Nanodrop

Treatment	TP1		TP2		TP3	
	Concentration (ng/µl)	OD 260/280	Concentration (ng/µl)	OD 260/280	Concentration (ng/µl)	OD 260/280
T4	211	2.09	-	-	-	-
T4	480	2.12	-	-	-	-
T4	157	2.13	-	-	-	-
12D6	-	-	84	2.05	199	1.99
12D6	-	-	68	2.07	247	1.97
12D6	-	-	79	2.1	179	2.07
16i	-	-	66	2.01	197	1.36
16i	-	-	90	2.1	169	2.13
16i	-	-	175	2.09	91	2.14
W_neg	-	-	109	2.01	117	2.01
W_neg	-	-	75	2.12	97	2.13
W_neg	-	-	86	2.03	162	2.14
W_pos	-	-	109	2.11	112	2.05
W_pos	-	-	75	2.02	108	2
W_pos	-	-	86	2.07	122	2.12

The results obtained showed that the isolated RNA was of good quality with OD260/280 between 1.7 and 2.13 and a concentration between 68 and 420 ng/µl and was sent to be sequenced.

RNA-sequencing and data preprocessing

The RNA samples were sent to be sequenced at the AgriLife Genomics and Bioinformatics Services Center (Texas A&M University). Sample libraries were sequenced using the Illumina NovaSeq. Sequence cluster identification, quality prefiltering, base calling, and uncertainty assessments were done in real-time using Illumina's NCS 1.0.2 and RFV 1.0.2 software with default parameter settings. Sequencer

cbcl basecall files were demultiplexed and formatted into .fastq files using bcl2fastq2 2.19.0 script configure BclToFastq.pl. A quality control (QC) was done and problems were corrected where possible and otherwise noted as such. Individual samples were then processed with FastQC version 0.11.7 to access the quality. Paired reads lengths were 100 bp and the run was done in two lanes.

Transcriptome analysis

Prior to alignment, the paired-end reads obtained during the sequencing were concatenated. The alignment was done using the STAR aligner software version 2.7.3a (Dobin et al., 2012) against the reference maize B73 genome RefGenv4 (http://www.ebi.ac.uk/ena/data/view/GCA_000005005.6), using the default settings. The estimation of the levels of gene expression was done by counting the reads that mapped to genes or exons. The gene expression levels was analyzed with the HTSeq software version 0.11.2 (Anders et al., 2015), with the default settings. Gene expression data obtained with the HTSeq served as an input file to study the differential gene expression (DEG) using the EdgeR in Rstudio environment. The default data filtering method used by edgeR package is the Counts Per Million (CPM). The EdgeR software version 3.28.0 and limma version 3.42.0 in R version 3.6.1 was used for the analysis.

The transcriptome analyses were conducted according to Hardoim et al. (2019) and Zheng et al. (2020) by comparing all the treatments at the second and third time points to the first time point with the EdgeR package in the Bioconductor version 3.11. The gene ontology analysis was performed using agriGO (http://systemsbiology.cau.edu.cn/agriGOv2/). The gene ontology classification was done

using the ShinyGO v0.61 software (Ge et al., 2019). The KEGG pathway analysis was performed using KEGG mapper (Kanehisa & Sato, 2020).

The data analysis was performed using Excel and the statistical software JMP Pro 15, accessed through the Virtual Open Access Lab of Texas A&M University. In order to understand the effect of the bacteria on plants under stress, we looked at genes uniquely or specifically expressed in the bacterial treated samples compared to plants under drought stress and to the healthy plants. To identify these genes, we used a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn).

CHAPTER III

RESULTS

Two PGPRs, a *Bacillus* 12D6, and an *Enterobacter* 16i, identified by Jochum et al. (2019) as conferring drought tolerance to maize and wheat, were used to study the time-course changes of maize seedlings transcriptomes. Comparisons were made with two controls, the absolute positive control, constituted by well-watered plants (Water positive or W_pos) during all the experiments, and the absolute negative control constituted by plants remaining completely under drought during the experiment (Water negative or W_neg). Three different time points were considered for data collection during the experiment, the first time point considered was the day of inoculation, 10 days post transplant, the second time point was 5 days post-inoculation (15 days post transplant) and the third time point was 9 days post-inoculation (19 days post transplanting). Plants' roots were collected for two purposes, RNA isolation for the transcriptome profiling and for the measurement of root system alteration caused by the two bacteria. Flag leaves of plants were also collected to determine their RWC.

Evaluation of the beneficial effect of the two PGPRs on seedlings under drought

In order to confirm the beneficial effects of the two bacteria, *Bacillus* 12D6 and *Enterobacter* 16i, on maize seedlings under drought stress prior to transcriptome profiling, three parameters were used: the drought phenotyping by assignment of drought score, the relative water content and the measurement of the alteration of the root system architecture. For the assignment of drought scores, results obtained (data not shown) show

that inoculation with the two bacteria significantly enhanced seedling tolerance to drought compared to the control plants, either non-inoculated or non-watered. Compared to the absolute non-watered control, the inoculated seedlings showed lower visible signals of drought stress: lodging, leaf drooping, leaf curling, leaf rolling, and wilting. The differences started being noticeable after 5 days of drought post-inoculation and after 9 days, the differences were very noticeable; with seedlings non-inoculated showing advanced wilting while inoculated plants were still healthy (Figure 3).

Figure 3: Effect of drought on flags leaves 9 dpi
16i= seedlings inoculated with *Enterobacter*, W_neg= plant under drought, 12D6= seedlings inoculated with *Bacillus*, W_pos=well-watered plants.

The beneficial effect of the bacterial inoculations was also observed on the leaf relative water content (Table 3). After 5 days of drought, inoculation with the two PGPRs significantly increased the RWC in the *Bacillus* 12D6 treated seedlings (96.12%) and the *Enterobacter* 16i treated seedlings (94.4%) in comparison to non-inoculated seedlings (82.8%). Nine days post-inoculation, this significance was clear between the seedlings

treated with the *Bacillus* 12D6 (84.97%) and the non-inoculated seedlings (64.37%), but between the seedlings inoculated with the *Enterobacter* 16i (76.47%) and the non-inoculated seedlings, the difference was not significant.

Table 3: Relative Water Content 5 and 9dpi

Treatments	Relative Water Content					
	Inoculation (TP1)		5 dpi (TP2)		9 dpi (TP3)	
	RWC	+/-SEM	RWC	+/-SEM	RWC	+/-SEM
T4	95.63	2.82	-	-	-	-
12D6	-	-	96.12A	1.59	84.97A	4.76
16i	-	-	94.94A	1.20	76.47AB	6.42
W_neg	-	-	82.80B	6.17	64.37B	1.98
W_pos	-	-	97.31A	0.35	81.85A	3.79

T4 = roots of non-inoculated plants taken the day of inoculation
The comparison of RWC of each treatment are done between time points
Means in the same column of each host plant with the same letter are not significantly different at P = 0.05.
SEM= standard error of mean.

Lastly, the beneficial effect of the two bacteria was also shown by their alteration of the root system architecture. After measurement with WhinRhizo (Figure 4) and analysis, the results showed that there were statistically significant differences between the inoculated seedlings and the absolute negative control in terms of root lengths and the number of tips produced 9 dpi. The difference was not statistically significant between treatments at 5 dpi. This significance in terms of root lengths and number of root tips is a sign of the beneficial effect of the two bacteria on seedlings under drought. The root length allows the plants to go deeper in the soil to have access to more water and the tips are largely responsible for the water uptake by the plant.

Figure 4: Results of roots measurement with WhinRhizo

Three roots samples from each treatment of the three time points were scanned using a flatbed scanner and analyzed using the WhinRhizo software to determine the roots length, number of tips and forks…

Transcriptome profiling

Library sequencing results

The RNA-seq libraries obtained after sequencing the roots of maize seedlings inoculated with two bacteria under stress in addition with plants well-watered and none-watered 0, 5 and 9 dpi generated 8.4 $x10^8$ reads total after concatenating the pair-end read sequences (Table 4), the details of the sequences for each sample is presented in the appendix A. At the first time point, the control treatment yielded 87 million reads. The second time point yielded 369 million reads with 89 million for the seedlings inoculated with the *Bacillus* 12D6 and 98 million for those inoculated with *Enterobacter* 16i. The third time point yielded a total of 381 million reads from which 98 million originated from 12D6 and 92 million for 16i. The absolute positive control yielded 93 million and 98 million reads respectively at time point 2 and time point 3 while the absolute negative control yielded respectively 88 million and 93 million.

Table 4: Sequencing reads per treatment obtained after sequencing and pre-preprocessing.

	Total sequence reads		
Treatments	**TP1**	**TP2**	**TP3**
T4(inoculation day)	87,221,837.00	-	-
12D6	-	89,118,822.00	98,023,470.00
16i	-	98,164,382.00	92,075,113.00
W_neg	-	88,384,288.00	93,008,803.00
W_pos	-	93,818,827.00	98,764,392.00
Total/TP	87,221,837.00	369,486,319.00	381,871,778.00
General total		838,579,935.00	

T4 = roots of non-inoculated plants taken the day of inoculation. These numbers represent the sum of the three replicates of each treatment during the same time point.

The mean quality score of the reads, which measures the mean quality value across each base position in the read, generated with the MultiQC software was high with a phred score between 35 and 36 (Figure 5 A). Concerning the Per Sequence Quality Scores, which shows if a subset of reads has poor quality, (Figure 5 B). These two figures showed the overall good quality of the sequence reads used during our study. The GC content of the reads was between 53 and 55 %.

A B

Figure 5: Means quality score (A) and per sequence quality score of reads (B) generated by using the sequencing results with the software MultiQC.
These figures are obtained by putting together all means quality score of all reads (Figure 5A) and all per sequence quality score of all reads (Figure 5B).When the majority of the quality score or per sequence quality score of reads are in the red or the yellow section of the histogram, the quality is considered not good and when it is in the green section, it is considered as good quality score.

Mapping to the maize reference genome:

The percentage of reads generated from each treatment that mapped to the B73 reference genome ranged from 90-94% (Table 5).

Table 5: Number of reads mapped to the maize reference genome for each sample, treatment and time points.

Sample ID	TP	Number of input reads	Unmapped reads	Uniquely mapped reads number	Mapping ratio
T4_1	1	31,105,172	1,446,440	28,738,155	92.39%
T4_2	1	26,596,359	766,794	24,845,966	93.42%
T4_3	1	29,520,306	1,227,955	27,320,393	92.55%
12D6_1	2	29,943,003	1,355,386	27,549,604	92.01%
12D6_2	2	28,280,490	1,344,616	26,059,759	92.15%
12D6_3	2	30,895,329	1,384,457	28,435,165	92.04%
16i_1	2	31,918,139	1,394,704	29,535,722	92.54%
16i_2	2	30,001,940	1,621,752	27,291,839	90.97%
16i_3	2	36,244,303	824,122	34,196,990	94.35%
W_neg_1	2	28,661,699	790,388	26,901,427	93.86%
W_neg_2	2	29,946,633	708,117	28,245,773	94.32%
W_neg_3	2	29,775,956	821,110	28,035,441	94.15%
W_pos_1	2	31,888,717	921,074	29,741,677	93.27%
W_pos_2	2	31,861,603	958,198	29,947,594	93.99%
W_pos_3	2	30,068,507	866,009	28,207,228	93.81%
12D6_1	3	32,505,196	1,096,080	30,095,046	92.59%
12D6_2	3	33,063,590	964,365	30,890,549	93.43%
12D6_3	3	32,454,684	922,127	30,452,170	93.83%
16i_1	3	31,486,887	875,860	29,472,352	93.60%
16i_2	3	32,096,641	1,655,064	29,457,596	91.78%
16i_3	3	28,491,585	1,959,634	25,737,103	90.33%
W_neg_1	3	31,286,023	861,166	29,456,604	94.15%
W_neg_2	3	29,842,843	815,676	27,900,173	93.49%
W_neg_3	3	31,879,937	935,160	29,852,057	93.64%
W_pos_1	3	37,516,314	1,206,150	35,119,874	93.61%
W_pos_2	3	29,516,126	1,407,845	27,269,251	92.39%
W_pos_3	3	31,731,952	1,173,862	29,393,986	92.63%

TP= time point

Transcriptome analysis

Comparisons of the transcriptome of the second and third time points with the first revealed a total of 17,293 differentially expressed genes. Of this, 11,812 DEGs were downregulated and 5,481 were upregulated. The results of the transcriptome analysis are presented in Table 6 below:

Table 6: Differentially expressed genes obtained after analysis

	differentially expressed	**Downregulated**	**Upregulated**
12D6_TP2 vs T4	707	528	179
12D6_TP3 vs T4	6700	4470	2230
W_neg_TP2 vs T4	236	194	42
W_neg_TP3 vs T4	4309	2559	1750
W_pos_TP2 vs T4	366	280	86
W_pos_TP3 vs T4	911	593	318
16i_TP2 vs T4	411	297	114
16i_TP3 vs T4	3653	2891	762

Up and down regulated genes refers in each case to the first term in the comparison relative to the second. Up regulated refers to an increase of the number of transcript and down regulated refers to a decrease of the number of transcripts. From now, 12D6_TP2 vs T4 will be refer only as 12D6_TP2 and same for the others.

The number of the differentially expressed genes was higher in the seedlings 9dpi than 5dpi (15,573 vs 1,720). The *Bacillus* treatment yielded the most DEGs with 7,407 genes followed by the seedlings of the absolute negative control (4,545 genes) and the *Enterobacter* treatment (4,064). The plants of the absolute positive control had the least number of DEGs at 1,277 genes (Figure 6). These results show that plants under drought

stress inoculated or not with bacteria yielded the most DEGs compare to the positive control.

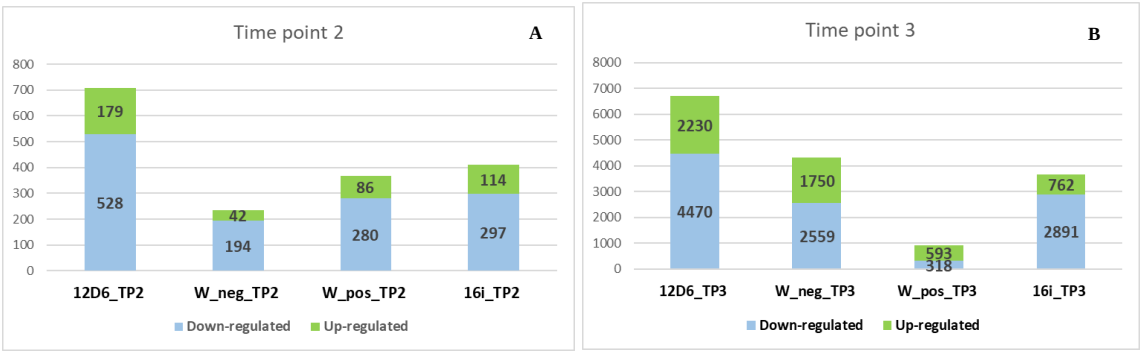

Figure 6: differentially expressed genes obtained after transcriptome analysis.
A: differentially expressed genes obtained 5 dpi showing the number of upregulated genes in green and downregulated genes in blue.
B: differentially expressed genes obtained 5 dpi showing the number of upregulated genes in green and downregulated genes in blue.

In order to have a better understanding of the beneficial effect of the two bacteria, data analysis was done to identify genes that were expressed uniquely as well as commonly between the different treatments. To do so, Venn diagrams were used at different time points to detect DEGs uniquely and commonly expressed by all the treatments.

Detection of genes expressed uniquely and commonly between the treatments at 5 dpi

At 5 dpi, in the *Bacillus* treatment (Figure 7), the Venn diagram shows that there were 45 genes commonly expressed between the bacterial treatment and the absolute negative control treatment. Between the bacterial treatment and the absolute positive control, 141 genes were expressed.

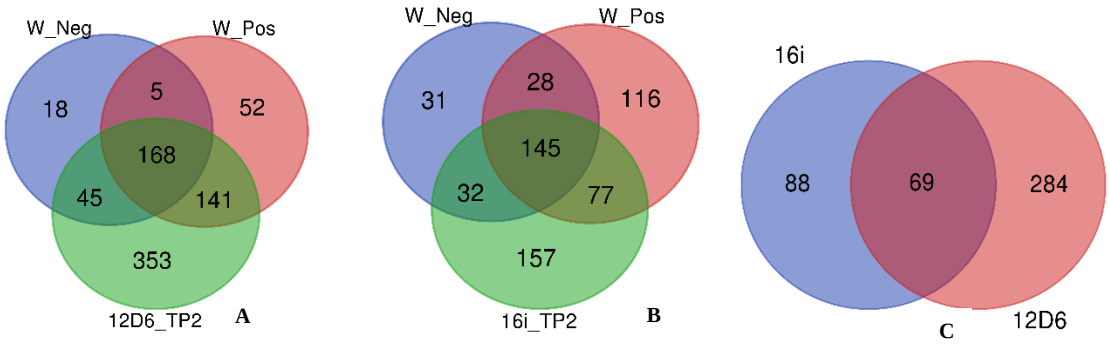

Figure 7: Venn diagram showing number of genes commonly or uniquely expressed by the bacterial inoculation and the controls (A and B), and comparison between uniquely expressed genes by the two bacteria.
The Venn diagram is obtained with http://bioinformatics.psb.ugent.be/webtools/Venn/. In Figure 7 A and B, the green part is showing the unique genes expressed by the two bacteria (green) in comparison with the absolute positive control (red) and absolute negative control (blue). These Figures also show the number of genes expressed commonly by the bacteria and the controls (overlapping colors). The Figure 7 C is showing the repartition of genes uniquely of commonly expressed by the two bacteria treatments.

The *Enterobacter* treatment (Figure 7B), had 32 and 77 DEGs in common with the positive and negative controls respectively. There were 353 and 157 genes uniquely expressed respectively by the *Bacillus* treatment and the *Enterobacter* treatment. Among these uniquely expressed genes, 88 were only expressed by the *Enterobacter* treatment, 284 only by *Bacillus* and 69 genes were common to both bacteria (Figure 7C).

Detection of genes uniquely and commonly expressed by the treatments 9 dpi

At nine days post-inoculation there were 2840 and 24 DEGs common to the *Bacillus* treatment and the negative and positive controls respectively (Figure 7A). In the *Enterobacter* treatment (Figure 8B), there were 1405 and 23 genes commonly expressed with the negative and positive controls respectively. Finally, among the uniquely

expressed genes by the two bacteria, 801 were common to both while 727 uniquely expressed by the *Enterobacter* and 2186 by the *Bacillus* (Figure 8C).

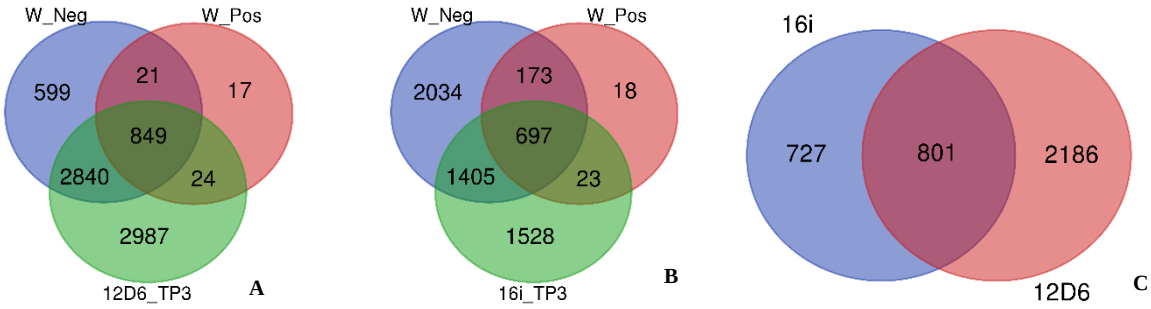

Figure 8: Venn diagram showing number of genes commonly or uniquely expressed by the bacterial inoculation and the controls (A and B), and comparison between uniquely expressed genes by the two bacteria.
The Venn diagram is obtained with http://bioinformatics.psb.ugent.be/webtools/Venn/. In figure 8 A and B, the green part is showing the unique genes expressed by the two bacteria (green) in comparison with the absolute positive control (red) and absolute negative control (blue). These figures also show the number of genes expressed commonly by the bacteria and the controls (overlapping colors). The figure 8 C is showing the repartition of genes uniquely of commonly expressed by the two bacteria treatments.

For the next step, we concentrate the analysis on genes that were uniquely expressed by seedlings inoculated with the two tested bacterial strains in comparison with the controls. The objective being to deepen our knowledge of the beneficial actions specific to each bacterium.

Functional classification using GO terms

The DEGs obtained during the experiment were assigned to GO terms under the three main categories of biological process, cellular component, and molecular function. The category cellular component was the most highly represented in terms of GO numbers matched. A subset of the results of the functional analysis obtained using ShinyGO is presented in Figures 9-14 (Appendix A for full report).

G.O. category Biological Process

In the category of Biological process, the GO terms highly enriched in the two bacteria treatments during both time points were “Response to stimulus”, “Regulation of metabolic process” and “Localization” (Figures 9 and 10). The term “Response to stimulus”, is defined as “any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus” in our case, it is drought. The term “Regulation of metabolic process” is defined as “any process that modulates the frequency, rate or extent of the chemical reactions and pathways within a cell or an organism”. The term “Localization” is “any process in which a cell, a substance, or a cellular entity, such as a protein complex or organelle, is transported, tethered to or otherwise maintained in a specific location”.

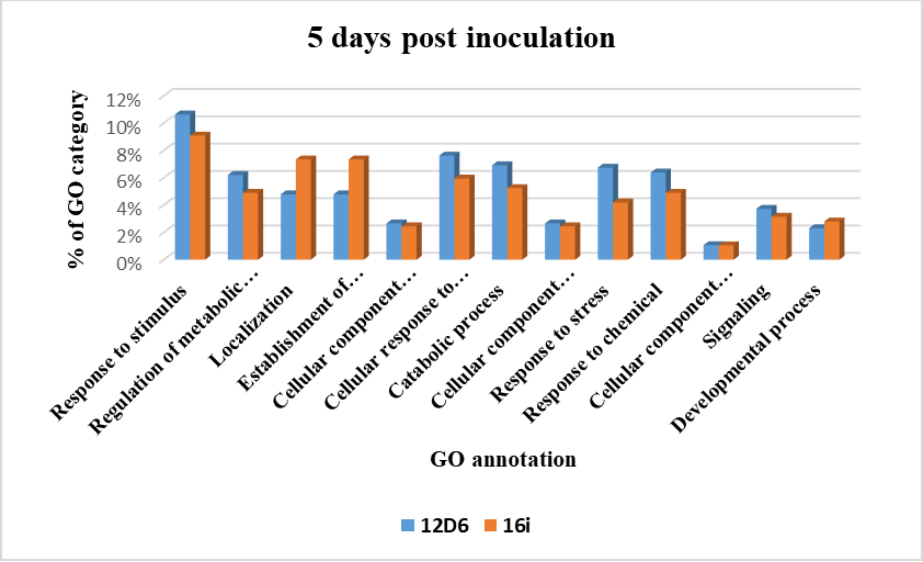

Figure 9 : A subset of functional analysis of GO category biological process for *Bacillus* 12D6 and *Enterobacter* 16i at 5 dpi

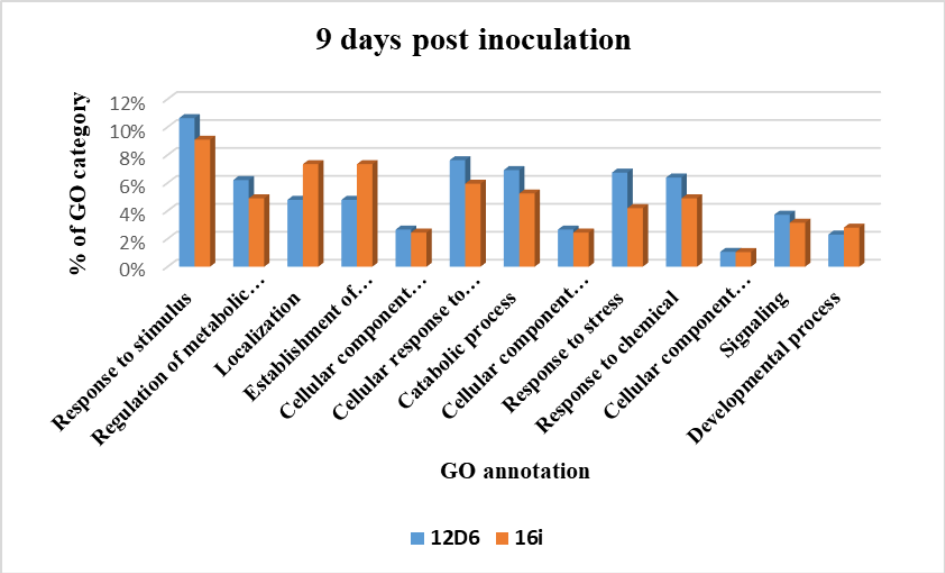

Figure 10: A subset of functional analysis of GO category biological process for *Bacillus* 12D6 and *Enterobacter* 16i at 9 dpi.
The diagram represents the number of GO in the biological process category of the two bacteria, blue for Bacillus 12D6 and orange for Enterobacter 16i. The name below each bar represents the specific GO category.

G.O. category Cellular Component

As in the biological process category, more GO terms were matched 9 dpi than 5 dpi and the *Bacillus* treatment yielded more GO terms compared to the *Enterobacter* treatment. The most represented terms are in this category are included “Cell periphery”, “Organelle part” and “Intracellular organelle part” (Figures 11 and 12). The Cell periphery is defined as “The part of a cell encompassing the cell cortex, the plasma membrane, and any external encapsulating structures”. The Organelle part is defined as “Organized structure of distinctive morphology and function”, while the Intracellular organelle part is understood as “Organized structure of distinctive morphology and function, occurring within the cell”.

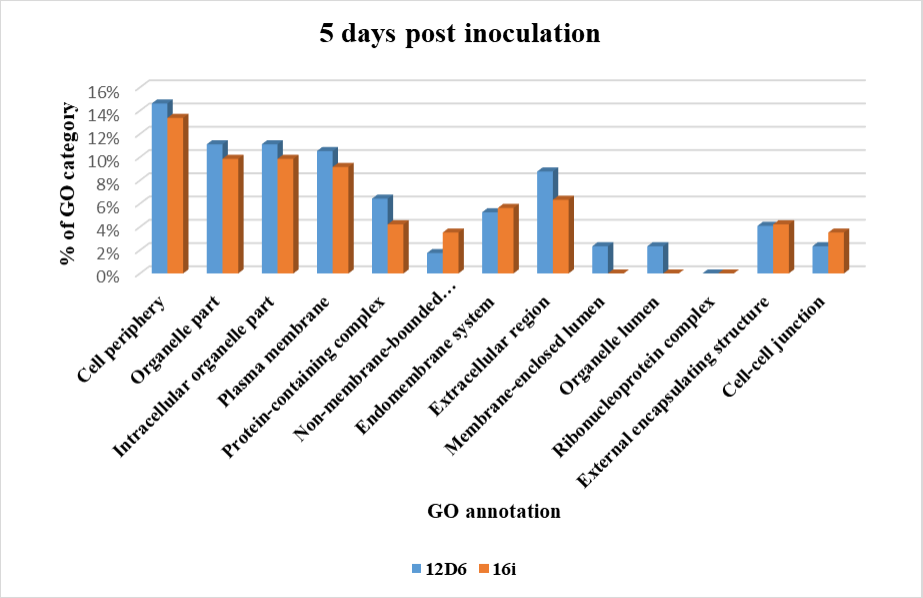

Figure 11: A subset of functional analysis of GO category Cellular component for *Bacillus* 12D6 and *Enterobacter* 16i at 5 dpi

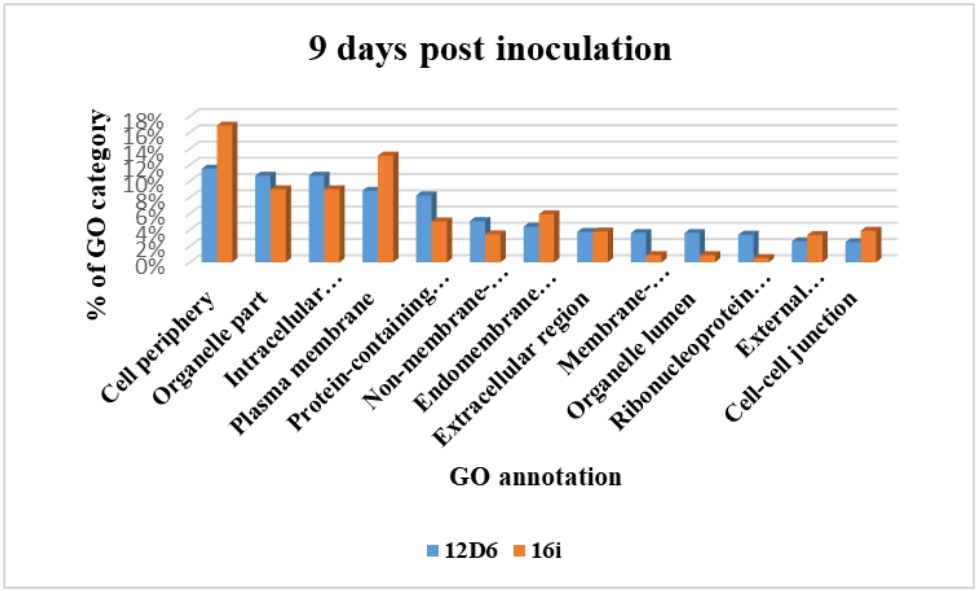

Figure 12: A subset of functional analysis of GO category Cellular component for Bacillus 12D6 and Enterobacter 16i at 9 dpi.
The diagram represents the number of GO in the Cellular Component category of the two bacteria, blue for Bacillus 12D6 and orange for Enterobacter 16i. The name below each bar represents the specific GO category.

G.O. category Molecular function

As in the previous two categories, there was more GO obtained at 9 dpi than at 5 dpi, and the *Bacillus* treatment similarly yielded more GO terms compared to the *Enterobacter* treatment. The most represented terms are "Small molecule binding", "Carbohydrate derivative binding" and "drug binding" (Figures 13 and 14). Small binding is defined as "Interacting selectively and non-covalently with a small molecule", and drug binding is defined as "Interacting selectively and non-covalently with a drug, any naturally occurring or synthetic substance, other than a nutrient, that, when administered or applied to an organism, affects the structure or functioning of the organism".

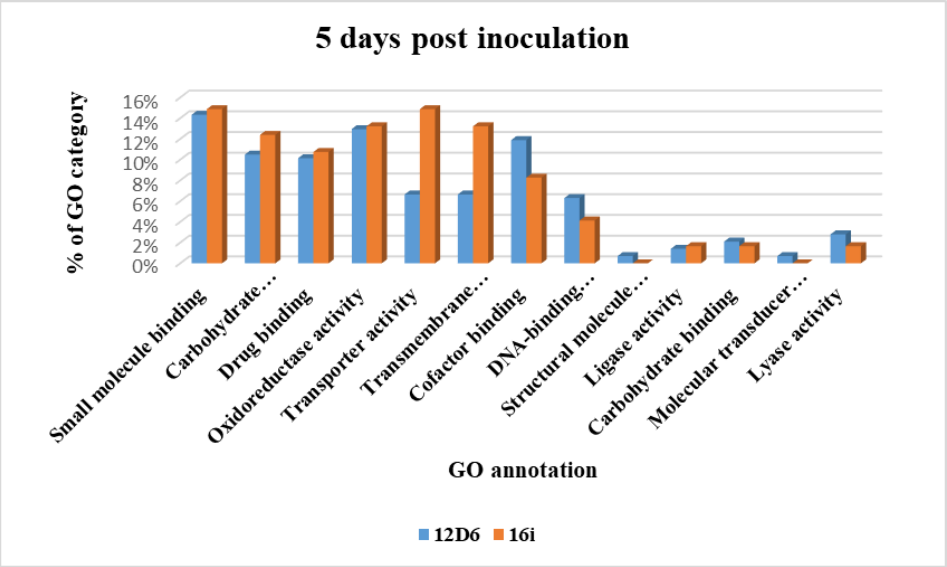

Figure 13: A subset of functional analysis of GO category Molecular function *Bacillus* 12D6 and *Enterobacter* 16i at 5 dpi.

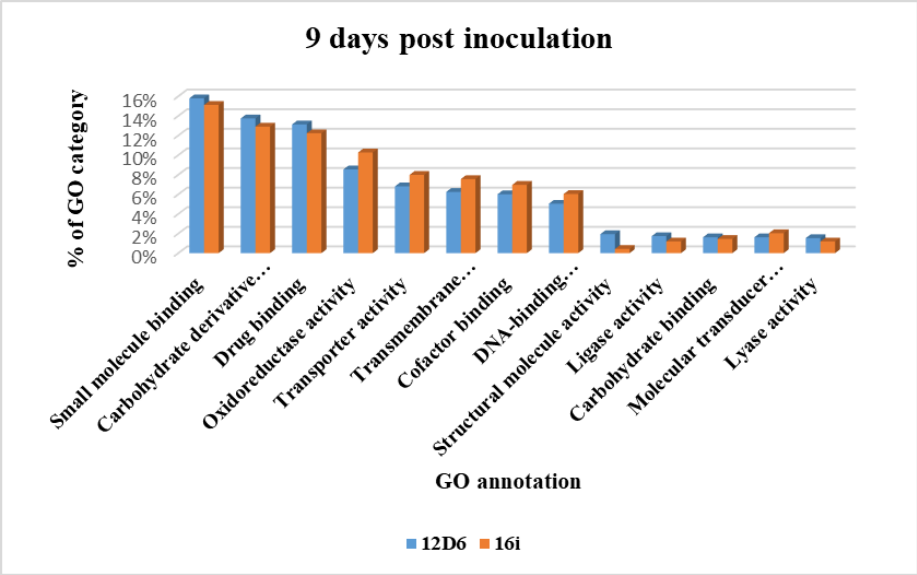

Figure 14: A subset of functional analysis of GO category Molecular function *Bacillus* 12D6 and *Enterobacter* 16i at 9 dpi.
The diagram represents the number of GO in the biological process category of the two bacteria. The name below each bar represents the specific GO category.

KEGG pathway analysis

KEGG pathways 5 dpi

The KEGG pathway analysis using KEGG mapper showed that genes expressed by the *Enterobacter* 16i inoculation were assigned to 42 different KEGG pathways, while those expressed by the *Bacillus* 12D6 inoculation were mapped to 54 different KEGG pathways. Large numbers of these DEGs were mapped to categories such as “Metabolic pathways”, “Biosynthesis of secondary metabolites”, “Plant hormone signal transduction” and “Plant-pathogen interaction”. In addition, the “MAPK signaling pathway” was represented in the *Enterobacter* treatment and not in the *Bacillus* treatment, while “Cysteine and methionine metabolism”, “Starch and sucrose metabolism”, “Biosynthesis

of amino acids", "alpha-Linolenic acid metabolism", and "Phenylpropanoid biosynthesis" were highly represented in the *Bacillus* treatment but not in the *Enterobacter* treatment. This suggests that the beneficial effect of these bacteria is linked to different pathways in the two bacteria.

The analysis of DEGs specific to each bacterium showed that the unique DEGs of the *Enterobacter* were mapped to 21 different pathways while those unique to the *Bacillus* were mapped to 43 KEGG pathways. The most represented categories common to both bacteria were "Metabolic pathways" and "Biosynthesis of secondary metabolites".

KEGG pathways 9 dpi

The *Enterobacter* 16i treatment at 9 dpi was assigned to 71 KEGG pathways and the *Bacillus* treatment to 116 different pathways. The most represented pathways were "Metabolic pathways", "Biosynthesis of secondary metabolites", "Phenylpropanoid biosynthesis", "Plant hormone signal transduction", "Starch and sucrose metabolism" and "Biosynthesis of amino acids". "Ribosome" and "Spliceosome" were overrepresented in the *Bacillus* treatment but not in the *Enterobacter* treatment.

The next step of the analysis will be focused on pathways that could provide a better understanding of the beneficial effect of the two bacteria. This focus will be on "Phenylpropanoid biosynthesis", "Plant hormone signal transduction", "Starch and sucrose metabolism", "Plant hormone signal transduction", and "Plant-pathogen interaction" (Appendix C for KEGG maps).

Analysis of genes differentially expressed linked to drought and plant-microbe interaction

DEGs related to Plant hormone signal transduction

In our study, a total of 38 DEGs related to the hormone metabolism pathways were obtained across the four treatments (Table 7).

Table 7: DEGs related to Plant hormone signal transduction

Gene ID	Gene Name/Description	Log_2FC 12D6 5dpi	Log_2FC 12D6 9dpi	Log_2FC 16i 5dpi	Log_2FC 16i 9dpi	KEGG definition
GRMZM2G005954	pco137346; uncharacterized protein LOC100191257	-1.898	-1.453	-	-	jasmonate ZIM domain-containing protein
GRMZM2G123119	putative AP2/EREBP transcription factor superfamily protein	-	-2.007	-	-	ethylene-responsive transcription factor 1
GRMZM2G004696	AUX3; IAA15-auxin-responsive Aux/IAA family member	-	0.601	-	-	auxin-responsive protein IAA
GRMZM2G163848	AUX34; AUX/IAA transcription factor	-	-0.797	-	-	auxin-responsive protein IAA
GRMZM2G047677	uncharacterized LOC100216590	-	-3.501	-	-	auxin-responsive protein IAA
GRMZM2G060723	TRAF18; hypothetical protein	-	-1.688	-	-	regulatory protein NPR1
GRMZM2G060290	bZIP70; putative bZIP transcription factor superfamily protein	-	-1.398	-	-	transcription factor TGA
GRMZM2G057959	PYL5; cyclase/dehydrase family protein	-	-3.537	-	-	abscisic acid receptor PYR/PYL family
GRMZM2G392125	xyloglucan endotransglucosylase/hydrolase protein 15 precursor	-	-2.253	-	-3.217	xyloglucan:xyloglucosyl transferase TCH4
GRMZM2G152796	AUX32; IAA14 - auxin-responsive Aux/IAA family member	-	-2.086	-	-2.820	auxin-responsive protein IAA
GRMZM2G030877	putative bZIP transcription factor superfamily protein	-	-1.782	-	-1.463	jasmonic acid-amino synthetase
GRMZM2G061005	uncharacterized protein LOC100384080	-	-2.701	-	-	jasmonic acid-amino synthetase
GRMZM2G125934	uncharacterized protein LOC100384496 isoform X1	-	-2.060	-	-	transcription factor TGA
GRMZM2G159134	putative bZIP transcription factor superfamily protein	-	0.632	-	-	jasmonic acid-amino synthetase
GRMZM2G312274	uncharacterized protein LOC103627198	-	-1.972	-	-1.952	SAUR family protein
GRMZM2G030710	auxin response factor 15	-	0.695	-	-	auxin response factor
GRMZM2G144224	abscisic acid receptor PYL4-like	-	-2.866	-	-	abscisic acid receptor PYR/PYL family

Table 7 Continued

Gene ID	Gene Name/Description	Log_2FC 12D6 5dpi	Log_2FC 12D6 9dpi	Log_2FC 16i 5dpi	Log_2FC 16i 9dpi	KEGG definition
GRMZM2G003558	ETHYLENE INSENSITIVE 3-like 1 protein	-	-2.43	-	-	ethylene-insensitive protein 3
GRMZM2G422419	auxin-responsive protein SAUR71	-	-2.192	-	-2.669	SAUR family protein
GRMZM2G420812	auxin-induced protein 15A	-	-1.968	-	-	SAUR family protein
GRMZM2G045057	auxin transporter-like protein 3	-	-1.604	-	-	auxin influx carrier (AUX1 LAX family)
GRMZM2G361993	uncharacterized protein LOC107436075	-	0.989	-	-	SAUR family protein
GRMZM2G319187	gpm84; uncharacterized protein LOC541630	-1.359	-	-	-	two-component response regulator ARR-A family
GRMZM2G130953	AUX22; hypothetical protein	0.996	-	1.017	-	auxin-responsive protein IAA
GRMZM2G035465	umc1460; uncharacterized protein LOC100274569 isoform 1	1.003	-	0.962	-	auxin-responsive protein IAA
GRMZM2G091276	uncharacterized protein LOC100381512	-1.338	-	-1.368	-	jasmonic acid-amino synthetase
GRMZM2G367348	pathogenesis-related protein PRB1-2	-	-	-2.997	-	pathogenesis-related protein 1
GRMZM2G060216	lg2; transcription factor LG2	-1.269	-	-1.386	-	transcription factor TGA
GRMZM2G180916	SnRK2.3 isoform 2	-	-	-	-1.177	serine/threonine-protein kinase SRK2
GRMZM2G128421	pco137466(715); Auxin-responsive protein IAA4	-	-	-	-1.089	auxin-responsive protein IAA
AC232238.2_FG004	transcription factor LG2-like isoform X1	-	-	-	-1.270	transcription factor TGA
GRMZM2G365162	indole-3-acetic acid-induced protein ARG7	-	-	-	-1.799	SAUR family protein
GRMZM2G001426	uncharacterized protein LOC103650192	-	-	-	0.944	DELLA protein
GRMZM2G061515	probable indole-3-acetic acid-amido synthetase GH3.1	-	-	-	-2.713	auxin responsive GH3 gene family

Gene ID, unique gene identifying number in the Maize Genetics and Genomics Database. Log_2 FC, fold change, is the ratio of intensities of upregulated or downregulated genes. All the negative fold change values (orange) means that the genes were downregulated. All the positive fold change values (blue) means the genes were upregulated. KEGG annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG), definition of the gene name

Nine genes pertaining to plant hormone signal transduction were found to be differentially expressed under *Bacillus* treatment of which 7 were downregulated and 2 upregulated, and five differentially expressed under *Enterobacter* treatment of which 3 were downregulated and 2 upregulated); 5 dpi. During this time point, four common DEGs were identified in the two bacterial treatments: two were linked to the Auxin pathway (GRMZM2G130953, GRMZM2G035465), one to the Jasmonic acid pathway (GRMZM2G091276), and one to the TGA transcription factor (GRMZM2G060216). The two genes linked to the auxin pathway were upregulated, and the others were downregulated.

A total of 23 genes were found to be differentially expressed in the *Bacillus* treatment of which, 20 were downregulated and 3 upregulated after 9 days of drought. Under the *Enterobacter* treatment, 11 DEGs were detected with one upregulated and 10 downregulated 9 dpi. Five DEGs were common to both bacteria during this time point: three linked to the Auxin pathway, one to the Jasmonic acid pathway, and one to the Xyloglucan pathway. In the *Bacillus* treatment, one gene (GRMZM2G005954) linked to the Jasmonic acid pathway was expressed by the bacterium treatment during both time points while in the *Enterobacter* treatment no common gene was expressed during both time points.

Among these DEGs related to the hormone transduction pathway, two were associated with the Ethylene pathway (both downregulated). Three genes related to ABA biosynthesis, were present only at 9 dpi, with two present in the *Bacillus* treatment and one in the *Enterobacter* treatment. In our study, five genes related to the TGA transcription

factors were represented, all expressed 9 dpi and downregulated. Three of these genes were specific to the *Bacillus* treatment, one linked to the *Enterobacter* treatment and one commonly expressed by both treatments.

In addition to these gene families, some other gene families were expressed at a low frequency across treatments. The DELLA protein associated with the Gibberellin pathway was only present in the *Enterobacter* treatment 9 dpi and was upregulated. There was also a gene linked to NPR1, a key regulator in the signal transduction pathway that leads to systemic acquired resistance, which was downregulated and present in *Bacillus* 9 dpi. One gene that was linked to the cytokinin pathway was present in the *Bacillus* treatment 5 dpi and downregulated. One gene was related to the Pathogenesis-related proteins was only present in the *Enterobacter* treatment 5 dpi.

DEGs related to phenylpropanoid biosynthesis

Phenylpropanoid biosynthesis associated genes were found downregulated in the *Bacillus* strain treatment 5 dpi, while no gene was found differentially in the *Enterobacter* treatment. However, 9 dpi, 23 DEGs related to this pathway were present in the *Bacillus* treatment, and 15 in the *Enterobacter* treatment. One gene (GRMZM2G063917) was expressed in the *Bacillus* treatment at 5 and 9 dpi, and 7 genes were commonly expressed by the two bacteria during the third time point (Table 8).

Most of the genes obtained during our study and linked to the Phenylpropanoid biosynthetic pathway were associated with the peroxidase pathway (23 out of 34). After the peroxidases, the second most represented is the Beta glucosidase pathway with 3 genes.

Table 8: DEGs related to phenylpropanoid biosynthesis

		Log 2 FC				
		12D6		16i		
Gene ID	Annotation	5dpi	9dpi	5dpi	9dpi	KEGG definition
#N/A	uncharacterized protein LOC100191153	-	-2.952	-	-	peroxidase
GRMZM2G108123	peroxidase 12 precursor	-	-2.937	-	-	peroxidase
GRMZM2G365774	uncharacterized protein LOC100194008	-	-3.221	-	-2.990	peroxidase
GRMZM2G108207	uncharacterized protein LOC100194034 precursor	-	-2.693	-	-	peroxidase
GRMZM2G150893	peroxidase 54 precursor	-	-2.105	-	-	peroxidase
GRMZM2G147245	putative cytochrome P450 superfamily protein	-	-1.115	-	-	trans-cinnamate 4-monooxygenase
GRMZM2G450233	gpm233; uncharacterized protein LOC100273479 precursor	-	-2.253	-	-	peroxidase
GRMZM2G118003	cl1052_1; uncharacterized protein LOC100274288 precursor	-	-1.177	-	-1.531	beta-glucosidase
GRMZM2G181259	uncharacterized protein LOC100279291	-	-0.858	-	-0.744	beta-glucosidase
GRMZM2G116902	uncharacterized protein LOC100280077 precursor	-	-1.740	-	-	peroxidase
GRMZM2G104109	peroxidase 1 precursor	-	-2.661	-	-3.127	peroxidase
GRMZM2G118345	phenylalanine ammonia-lyase	-	-1.203	-	-	phenylalanine ammonia-lyase
GRMZM2G063917	phenylalanine ammonia-lyase	-1.464	-1.118	-	-	phenylalanine ammonia-lyase
GRMZM2G134947	peroxidase N	-	-2.403	-	-	peroxidase
GRMZM2G061776	peroxidase 45	-	-3.391	-	-	peroxidase
GRMZM2G437207	peroxidase 2-like	-	-3.660	-	-3.248	peroxidase
GRMZM2G168073	peroxidase 2-like	-	-5.952	-	-	peroxidase
GRMZM2G024234	peroxidase 2-like	-	-7.263	-	-	peroxidase
GRMZM2G117365	peroxidase 2	-	-4.972	-	-	peroxidase
GRMZM2G097934	peroxidase 4	-	-7.044	-	-	peroxidase
GRMZM2G410175	uncharacterized protein LOC107548102 precursor	-	-3.354	-	-3.732	peroxidase

Table 8 Continued

		Log 2 FC				
		12D6		16i		
Gene ID	Annotation	5 dpi	9 dpi	5 dpi	9 dpi	KEGG definition
GRMZM2G133475	peroxidase 66 precursor	-	-5.077	-	-3.775	peroxidase
GRMZM2G473711	gla3; beta-glucanase precursor	-	-3.484	-	-	beta-glucosidase
#N/A	uncharacterized protein LOC100192105 precursor	-1.446	-	-	-	peroxidase
GRMZM2G041308	peroxidase 72 precursor	-2.854	-	-	-	peroxidase
GRMZM2G138074	putative cytochrome P450 superfamily protein	-	-	-	1.203	5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase
GRMZM2G020523	uncharacterized protein LOC100272496 isoform X1	-	-	-	1.882	peroxidase
GRMZM2G100158	putative cytochrome P450 superfamily protein	-	-	-	1.292	ferulate-5-hydroxylase
GRMZM2G127251	uncharacterized protein LOC100274269	-	-	-	1.378	shikimate O-hydroxycinnamoyltransferase
AC210003.2_FG004	pco083783; uncharacterized protein LOC100274427 precursor	-	-	-	-1.379	peroxidase
GRMZM2G044092	TIDP3759; uncharacterized protein LOC100280071	-	-	-	-0.916	beta-glucosidase
GRMZM2G116823	peroxidase A2 isoform X2	-	-	-	3.063	peroxidase
GRMZM2G063435	peroxidase 44-like precursor	-	-	-	-2.299	peroxidase

Gene ID, unique gene identifying number in the Maize Genetics and Genomics Database. Log_2 FC, fold change, is the ratio of intensities of upregulated or downregulated genes. All the negative fold change values (orange) means that the genes were downregulated. All the positive fold change values (blue) means the genes were upregulated. KEGG annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG), definition of the gene name

DEGs related to starch and sucrose metabolism

Eight DEGs associated with the Trehalose-6-phosphate phosphatases in our study, (Table 9); in which, 5dpi, three were present in the *Bacillus* treatment, all downregulated, and one in the *Enterobacter* treatment, also downregulated. Nine days post-inoculation; four genes linked to the Trehalose-6-phosphate phosphatases were present in the *Bacillus* treatment and two in the *Enterobacter* treatment. In the *Bacillus* treatment, all four genes were downregulated, and in the *Enterobacter* treatment, between the two genes differentially expressed, one was upregulated and one downregulated. Another important starch and sucrose metabolism pathway representative was the enzyme beta-glucosidase, which was linked to four DEGs all expressed 9 dpi. All these DEGs were downregulated, 2 common to both bacteria and one specific to each bacterium.

Concerning the sucrose metabolism pathway, our study yielded that, the four DEGs linked to this pathway were all Sucrose phosphate synthase. There was only one gene linked to the sucrose metabolism expressed 5 dpi by the *Bacillus* treatment and it was downregulated. There were three DEGs expressed at 9 dpi, of which two were upregulated and one downregulated. The downregulated gene of the *Bacillus* treatment was also expressed and downregulated in the *Enterobacter* treatment. Finally, in our study, the only gene involved in the starch break down, Beta amylase, was present only in the *Bacillus* treatment 5 dpi and was highly upregulated.

Table 9: DEGs related to starch and sucrose metabolism

		Log$_2$FC				
		12D6		16i		
Gene ID	Gene Name/Description	5dpi	9dpi	5dpi	9dpi	KEGG Annotation
GRMZM2G008263	GBSSIIa; granule bound starch synthase IIa precursor	-	1.590	-	-	granule-bound starch synthase
GRMZM2G074158	pho1; alpha-1,4-glucan phosphorylase	-	0.961	-	-	glycogen phosphorylase
GRMZM2G111324	putative O-Glycosyl hydrolase superfamily protein precursor	-	-1.529	-	-1.359	glucan endo-1,3-beta-glucosidase
GRMZM2G080354	uncharacterized protein LOC100272656	-	-1.567	-	-	trehalose 6-phosphate phosphatase
GRMZM2G118003	cl1052_1; uncharacterized protein LOC100274288 precursor	-	-1.177	-	-1.531	beta-glucosidase
GRMZM2G181259	uncharacterized protein LOC100279291	-	-0.858	-	-0.744	beta-glucosidase
GRMZM2G140614	glucose-6-phosphate isomerase	-	-0.740	-	-	glucose-6-phosphate isomerase
GRMZM2G122231	gpm600; uncharacterized protein LOC100281927	-1.637	-1.480	-	-	trehalose 6-phosphate synthase/phosphatase
GRMZM2G143747	uncharacterized protein LOC100381926	-	-2.245	-	-2.592	endoglucanase
GRMZM2G055331	putative sucrose-phosphate synthase family protein	-	0.887	-	-	sucrose-phosphate synthase
GRMZM5G890599	probable trehalose-phosphate phosphatase 10	-	-3.340	-	-	trehalose 6-phosphate phosphatase
GRMZM5G856653	hexokinase-6	-	1.299	-	-	hexokinase
GRMZM2G366659	probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 10	-	-1.579	-	-	trehalose 6-phosphate synthase/phosphatase
GRMZM2G045171	sucrose synthase 6	-	-2.791	-	-3.353	sucrose synthase
GRMZM2G051677	frk2; fructokinase-2	-	-1.550	-	-	fructokinase
GRMZM2G152908	sus1; sucrose synthase 2	-	0.971	-	-	sucrose synthase
GRMZM2G473711	gla3; beta-glucanase precursor	-	-3.484	-	-	beta-glucosidase
GRMZM2G455642	pco100822; uncharacterized protein LOC100216867 precursor	-	-	-	-1.530	endoglucanase
GRMZM6G738249	uncharacterized protein LOC100273093	-	-	-	0.945	trehalose 6-phosphate phosphatase
GRMZM2G044092	TIDP3759; uncharacterized protein LOC100280071	-	-	-	-0.916	beta-glucosidase
GRMZM2G097207	glucan endo-1,3-beta-glucosidase 6 precursor	-	-	-	-0.747	glucan endo-1,3-beta-glucosidase

Table 9 Continued

		Log$_2$FC				
		12D6		16i		
Gene ID	**Gene Name/Description**	**5dpi**	**9dpi**	**5dpi**	**9dpi**	**KEGG definition**
GRMZM2G048165	endoglucanase 23-like precursor	-	-	-	-2.687	Endoglucanase
GRMZM2G151044	uncharacterized protein LOC100381600	-	-	-	-2.353	trehalose 6-phosphate phosphatase
GRMZM2G527891	putative trehalose phosphatase/synthase family protein	-1.359	-	-	-	trehalose 6-phosphate synthase/phosphatase
GRMZM2G450125	pco104637; beta-amylase	3.732	-	-	-	beta-amylase
GRMZM2G118462	probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 7	-1.153	-	-1.047	-	trehalose 6-phosphate synthase/phosphatase
GRMZM2G097641	sucrose-phosphatase 2	-2.019	-	-	-	sucrose-6-phosphatase

Gene ID, unique gene identifying number in the Maize Genetics and Genomics Database (Maize GDB). Gene name/description, name or description of the gene identified by the given Gene ID. Log$_2$ FC, fold change, is expressed as the ratio of intensities of upregulated or downregulated genes between inoculated seedlings under drought stress treatments and the control at the beginning of the experiment. All the negative fold change values (orange) means that the genes were downregulated. All the positive fold change values (blue) means the genes were upregulated. KEGG annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG), definition of the gene name.

DEGs related to plant-pathogen interaction

Four DEGs (2 upregulated and 2 downregulated) from the *Bacillus* treatment were related to the plant-pathogen interaction pathway, and 3 DEGs (1 upregulated and 2 downregulated) were linked to the *Enterobacter* treatment 5 dpi. Among these DEGs, 2 were common (GRMZM2G027351, GRMZM2G080041). At 9 dpi, 14 DEGs (2 upregulated, 12 downregulated) were linked to this pathway in the *Bacillus* treatment and 2 (all downregulated) to the *Enterobacter* treatment. In addition, one gene (GRMZM2G003501) was common to both bacteria (Table 10).

Table 10: DEGs related to plant-pathogen interaction

		Log 2 FC				
		12D6		16i		
Gene ID	Gene Name/Description	5dpi	9dpi	5dpi	9dpi	KEGG definition
GRMZM2G061447	Pti1b; putative Pti1-like kinase	-	-0.540	-	-	pto-interacting protein 1
GRMZM2G003501	g14A	-	-1.686	-	-1.792	3-ketoacyl-CoA synthase
GRMZM2G148087	TIDP2949; uncharacterized protein LOC100279301	-	-1.743	-	-	WRKY transcription factor 33
GRMZM2G444621	TIDP3279; uncharacterized protein LOC100280399	-	-1.673	-	-	calcium-binding protein CML
GRMZM2G023037	si660042c08; uncharacterized protein LOC100281197	-	-0.697	-	-	cyclic nucleotide gated channel, plant
GRMZM2G343024	pto kinase interactor 1	-	-0.585	-	-	pto-interacting protein 1
GRMZM2G365035	cl31449_1; uncharacterized protein LOC100284681	-	-0.579	-	-	calcium-dependent protein kinase
GRMZM2G031329	uncharacterized protein LOC100381981	-	-1.790	-	-	calcium-binding protein CML
GRMZM2G058305	calcium-dependent protein kinase 1	-	-0.647	-	-	calcium-dependent protein kinase
GRMZM2G340313	putative calcium-binding protein CML19	-	-3.474	-	-	calcium-binding protein CML
AC233893.1_FG003	3-ketoacyl-CoA synthase 6-like	-	-1.852	-	-	3-ketoacyl-CoA synthase
GRMZM2G003059	calcium-dependent protein kinase 8-like	-	2.079	-	-	calcium-dependent protein kinase
GRMZM2G112165	heat shock protein 81-1	-	1.219	-	-	molecular chaperone HtpG
GRMZM2G426046	putative calcium-binding protein CML19	-	-1.735	-	-	calcium-binding protein CML
GRMZM2G031963	probable WRKY transcription factor 34	-	-	-	-2.110	WRKY transcription factor 2
GRMZM2G168304	uncharacterized protein LOC100382314	2.776	-	-	-	3-ketoacyl-CoA synthase

Table 10 Continued

		Log 2 FC				
		12D6		16i		
Gene ID	**Gene Name/Description**	**5dpi**	**9dpi**	**5dpi**	**9dpi**	**KEGG definition**
GRMZM2G321239	IDP2562; uncharacterized protein LOC100384476	-1.521	-	-	-	calcium-dependent protein kinase
GRMZM2G027351	calcium-dependent protein kinase 11	2.475	-	2.430	-	calcium-dependent protein kinase
GRMZM2G080041	LRR receptor-like serine/threonine-protein kinase FLS2	-3.161	-	-3.273	-	LRR receptor-like serine/threonine-protein kinase FLS2
GRMZM2G367348	pathogenesis-related protein PRB1-2	-	-	-2.997	-	pathogenesis-related protein 1

Gene ID, unique gene identifying number in the Maize Genetics and Genomics Database (Maize GDB). Gene name/description, name or description of the gene identified by the given Gene ID. Log_2 FC, fold change, is expressed as the ratio of intensities of upregulated or downregulated genes between inoculated seedlings under drought stress treatments and the control at the beginning of the experiment. All the negative fold change values (orange) means that the genes were downregulated. All the positive fold change values (blue) means the genes were upregulated. KEGG annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG), definition of the gene name.

CHAPTER IV

DISCUSSION

In order to study the transcriptome of maize seedlings inoculated with two different PGPRs under drought, an experiment was conducted. Maize seedlings aged 10 days were inoculated with a *Bacillus* and an *Enterobacter* strain and subjected to drought. Five and nine days after inoculation, drought phenotyping was conducted to monitor the beneficial effects of the two bacteria on the seedlings in comparison with an absolute positive control, well-watered during the experiment, and absolute negative control not watered at all during the experiment. Transcriptome profiling exercise was also conducted to detect genes differentially expressed by the seedlings inoculated with the two bacteria.

During the study, the roots system architecture was seen as a whole and the effect of the bacteria on the different roots part was not separated. The study being designed to look at the effect of bacteria strains on the plants, by trying to look specifically to each root part; it will not allow assessing the overall effect of the bacteria on the plant's roots. In addition, the study was conducted on the early stages of the maize (between 0 and 19 days) where the differences between root parts are not easily assessable.

Effect of the bacterial inoculation on plants under drought

The results obtained show that there was more water retained in leaves of seedlings inoculated with the *Bacillus* 12D6 strain than in the *Enterobacter* 16i strain, compared to plants under drought without inoculation. These results are similar to those reported by Sandhya et al. (2010) and Skz et al. (2018), who reported the ability of PGPR, namely *P.*

putida to enhance the water content of an inoculated plant under drought compared to the non-inoculated control. In the present study, five days post-inoculation, the bacterial treatment allowed the inoculated seedlings to retain water as much as the healthy seedlings, but after 9 days of drought, it was only the *Bacillus* treatment that allowed seedlings to retain water as much as the healthy seedlings. The inoculation with the *Enterobacter* after 9 days of the drought was able to allow seedlings to retain water but not as statistically significant as the *Bacillus* inoculation.

Concerning the effects of the inoculation on the root system architecture, our study showed that there were statistically significant differences in terms of roots lengths and number of tips between the inoculated seedlings than those uninoculated under drought. The bacterial inoculation increased these parameters in comparison to the control under drought. These results are concordant with those reported by Jochum et al. (2019) working on the same bacteria. The root lengths allow the plants to go deeper in the soil to have access to more water and the tips are largely responsible for the water uptake.

Our results also show that 5 dpi, the Aux/IAA responsive genes were upregulated in both bacteria treatment suggesting production of IAA in the plants under drought inoculated with the two bacteria. The study by Jochum et al. (2019) has also reported the ability of the two bacteria to produce this hormone exogenously. The production of this hormone was only present in the *Bacillus* treatment 9 dpi where one gene related to this hormone was upregulated while in the Enterobacter treatment, all genes related to Aux/IAA were downregulated. These results suggest that the production of IAA is done

early in plants in response to drought and that the induction of IAA production is high in the seedlings inoculated with the *Bacillus* than those inoculated with the *Enterobacter.*

In conclusion, we were able to confirm the beneficial effect of the two bacteria on maize seedlings under drought. The inoculation with the two bacteria was able to allow seedlings to retain more water and to be able to explore more soil surface with an ability to increase the uptake of water. Finally, it shall be noted that the *Bacillus* treatment was better in conferring drought tolerance than the *Enterobacter* treatment.

DEGs related to hormone metabolism

Many plant hormones, such as Ethylene, Abscisic Acid, Jasmonates, Salicylic Acid, Cytokinins, Auxin, and Brassinosteroids have been reported to be involved in stress signaling in plants (Munné-Bosch & Müller, 2013). In the present study, 38 DEGs related to the hormone metabolism pathways expressed by the two bacterial treatments during the two time points.

Among these genes associated with the hormone metabolism in plants, eight were linked to the Jasmonic acid hormone transduction pathway. Jasmonic acid (JA) hormone transduction pathway plays a significant role in the alleviation of a plant's abiotic stresses like drought (Ali & Baek, 2020). Four of these genes were linked to the Jasmonate ZIM (zinc-finger inflorescence meristem) domain (JAZ) proteins, which are involved in the responses to plant pathogens, abiotic stresses and are important signaling molecules of the JA pathway (Liu et al., 2017). The JAZ proteins act like transcriptional repressors that inhibit the expression of jasmonate-responsive genes (Ali & Baek, 2020). In our study, the genes related to the JAZ proteins were all downregulated and only expressed by the

Bacillus treatment during both time points. This result suggests that the *Bacillus* inoculation to maize seedlings induces JA biosynthesis in plants by repressing the activity of JAZ responsive proteins. Based on the number of genes downregulated 5 dpi compared to those expressed 9 dpi (4 vs 1), we can assume that the activity of JA responsive genes in the early stage of the drought was induced by the *Bacillus* inoculation compared to the advanced days of the drought. This early activity of JA responsive genes suggests that the beneficial action of the *Bacillus* can be linked to the production of JA.

Another important JA pathway representative, the jasmonic acid-amino synthetase JAR1, had 4 differentially expressed genes in our study. Two downregulated genes expressed by the *Enterobacter* treatment, one observed 5 dpi, and the other one 9 dpi. In the *Bacillus* treatment, 5 dpi, there was one gene downregulated linked to JAR1 while two genes, one was upregulated and the other one downregulated was related to JAR1 9 dpi. JAR1 is a JA biosynthetic gene and its downregulation in the *Enterobacter* treatment can be associated with a decrease in the activity of JA responsive genes in seedlings inoculated with this bacterium. In the *Bacillus* treatment, the up and downregulation can be linked to the maintenance of the perfect balance of the activity of JA responsive genes in the plant. The inoculation of *A. thaliana* with *Pseudomonas chlororaphis* O6 under drought was reported to favor the production of JA (Cho et al., 2013). In our study, there was more JA producing gene expressed overall in the *Bacillus* treatment than the *Enterobacter* treatment and this can be suggested as an explanation of the ability of the *Bacillus* to alleviate more drought in the seedlings compared to the *Enterobacter*.

Ethylene is crucial for many developmental processes and a key mediator of biotic and abiotic stress responses in plants. The Ethylene Response Factor (ERF) superfamily is known to play an important role in plant adaptation to biotic and abiotic stresses, such as drought (Munné-Bosch & Müller, 2013). In our study, two genes related to the Ethylene pathway, the *ethylene-responsive transcription factor 1* and the *ethylene-insensitive protein 3,* were expressed and downregulated in the *Bacillus* treatment 9 dpi. Skz et al. (2018) after inoculating maize seedlings with *P. putida* strain FBKV2 and Mayak et al. (2004) after inoculating tomato and pepper with *Achromobacter piechaudii* ARV8 under drought have also reported a decrease in the abundance of genes related to the Ethylene pathway. The fact that the same pattern was observed in the *Bacillus* treatment 9 dpi can also explain its ability to confer drought tolerance after a long period of drought. The genes related to the Ethylene pathway were not differentially expressed in the *Enterobacter* treatment suggesting that the beneficial action of these bacteria was not linked to the downregulation of the Ethylene pathway.

The most represented hormone pathway triggered in our study was the Auxin pathway with 15 representative genes. The auxin-responsive genes classes include the *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) family, the *auxin response factor* (*ARF*) family, *small auxin upregulated RNA* (*SAUR*), and the *auxin-responsive Gretchen Hagen3* (*GH3*) family (Luo et al., 2018). Auxin has been associated with the negative regulation of drought tolerance and its content is reduced under drought conditions (Xie et al., 2003, Vargas et al., 2014). While Vargas et al. (2014) have reported in wheat that drought stress tolerance was linked to a decrease in IAA content in the plant, Jung et al. (2015) have

reported that the overexpression of an *Aux/IAA* gene in rice was able to improve tolerance to drought stress. According to Paponov et al. (2008), auxin-induced *SAURs* are most highly expressed in shoots, whereas several auxin-repressed and nonresponsive *SAURs* are preferentially expressed in roots.

Five days post-inoculation; two of the same genes related to the Auxin pathway were upregulated in the two bacteria. These genes were linked to the Aux/IAA protein family, representing a large family of auxin co-receptors and transcriptional repressors that play a central role in auxin signaling (Salehin et al., 2019). Based on the work by Jung et al. (2015) on rice, we can surmise that the beneficial activity of the two bacteria can be associated 5 dpi to the induction of auxin-responsive genes in inoculated plants.

Ten genes related to the Auxin pathway were expressed by the *Bacillus* treatment 9dpi; among them, four were linked to the Aux/IAA family (three downregulated and one upregulated), four were linked to the *SAUR* family (three downregulated and one upregulated), one linked to auxin influx carrier downregulated and one to the auxin response factor, upregulated. Considering studies by Vargas et al. (2014) and Paponov et al. (2008) and based on the number of downregulated DEGs linked to Aux/IAA and *SAUR* family genes expressed by the seedlings inoculated with the *Bacillus* 9 dpi, it can be surmised that the activity of genes responsible of the production of auxin was decreased at an advanced drought state. In the *Enterobacter* treatment, 9 dpi, there were six genes related to the Auxin pathway. Three were related to the *SAUR* family, two to *Aux/IAA* and, one to the *GH3 family*. In conclusion, 9 dpi, the inoculation with the two bacteria was linked to a decreased activity of auxin responsive genes in the seedlings. This decrease in

the auxin-responsive genes after inoculation with PGPRs are similar to the results reported by Skz et al. (2018) and Vargas et al. (2014). Their studies reported that the inoculation with beneficial microbes respectively to maize and sugarcane resulted in the downregulation of genes encoding GH3, GH3.8, SAUR56, and AUX/IAA.

Abscisic acid is considered the most crucial phytohormone conferring abiotic stress tolerance in plants; it is also known to promote partial or complete closure of stomata. In the ABA signal transduction pathways, there are three important protein classes: (i) the pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor (*PYR/PYL/RCARs*) which are suggested to be the ABA receptors; (ii) *protein phosphatase 2Cs (PP2Cs)* acting as negative regulators; and, (iii) SNF1-related protein kinase 2 s (*SnRKs*) acting as positive regulators (Sah et al., 2016). In our study, the ABA regulators, PYL4 and PYL5 were only present in the *Bacillus* treatment 9 dpi and were downregulated whereas the *SnRKs* were only present in the *Enterobacter* treatment 9 dpi and was downregulated. These results are similar to those reported by Vargas et al. (2014) who described the same downregulation pattern of ABA-responsive genes after inoculation with a PGPR. Our results were also similar to those of Skz et al. (2018) concerning the PYL4 and PYL5 genes which were downregulated but not similar concerning *SnRKs* which were found upregulated in their case.

The TGA transcription factors are a subfamily of bZIP group D and they play vital roles in various biological processes, like growth and development, responses to pathogens, and abiotic stress (Li et al., 2019). In our study, five genes related to the TGA transcription factors were represented, which all expressed 9 dpi and downregulated.

Three of these genes were specific to the *Bacillus* treatment, one linked to the *Enterobacter* treatment and one expressed by both treatments. Among the genes related to TGA reported by our study, two were uncharacterized, two linked to LG2 TF, and one linked to bZIP70. These three last genes encode basic leucine zipper protein (Walsh et al., 1998). According to Xiang et al. (2008) and Chen et al. (2012), the overexpression of basic leucine zipper protein in rice was able to confer drought tolerance to this plant. The downregulation of these genes obtained in our study is not concordant to those reported by two previous authors.

DEGs related to Carbohydrates metabolism

The catabolism of carbohydrates is very crucial for plant survival by providing important saccharides and energy required for cell function. It is suggested that the differences in gene expression associated with carbohydrate metabolism lead to the regulations undergone by plants during drought stress (Min et al., 2016).

According to Lin et al. (2019) the Trehalose-6-phosphate phosphatases (TPPs), encoded by members of the *TPP* gene family, improve plants' tolerance to drought. Trehalose is important for plants because it acts as a protectant to stabilize membranes and proteins in certain plants allowing them to survive during dehydration–rehydration cycles. In our study, eight DEGs were related to the TPP pathway. Five days post-inoculation, three genes were present in the *Bacillus* treatment and all downregulated while one was present in the *Enterobacter* treatment, also downregulated. Nine days post-inoculation, four genes were present in the *Bacillus* treatment, and two in the *Enterobacter* treatment. In the *Bacillus* treatment 9 dpi, all four genes were downregulated, and in the *Enterobacter*

treatment, of the two genes expressed, one was upregulated and one downregulated. This overall downregulation of the TPP genes observed in our study in the two bacteria in both time points is inconsistent with the observation by Nuccio et al. (2015) that the overexpression of the gene encoding a rice TPP in developing maize ears increases maize yield under drought conditions. On the other hand, the upregulation of one gene in the *Enterobacter* treatment 9 dpi observed can be suggested as one factor allowing it to confer drought tolerance.

The enzyme beta-glucosidase is associated with many processes in plants, like the catalysis of β-d-glucosidic bond hydrolysis that leads to the release of glucose, and the timely response to biotic and abiotic stresses by the activation of phytohormones and defense compounds (Gómez-Anduro et al., 2011). In our study, four DEGs were linked to the beta-glucosidase family, and all expressed 9 dpi. All these DEGs were downregulated, two common to both bacteria and one specific to each bacterium. The fact that these genes were downregulated in our study and cannot allow the activity of gene releasing glucose, the activation of phytohormones and defense compounds as previously reported by (Gómez-Anduro et al., 2011) means that the beneficial effect of the two bacteria was not linked to the activity of the beta-glucosidase genes.

The biosynthesis of sucrose is suggested to help the plants adjust and maintain homeostasis, allowing the continuation of normal cellular functions despite drought stress, as a result of sucrose hydrolysis; there is an accumulation of glucose and fructose serving as a source for glycolysis and the synthesis of other saccharides (Krasensky & Jonak, 2012, Skz et al., 2018). Our study revealed that four DEGs linked to sucrose metabolism

were all Sucrose phosphate synthase (SPS), involved in sucrose biosynthesis in plants. There was only one gene expressed 5 dpi by the *Bacillus* treatment and it was downregulated. There were three DEGs expressed at 9 dpi; 2 were upregulated and 1 downregulated. The downregulated gene of the *Bacillus* treatment was also expressed and downregulated in the *Enterobacter* treatment. The decrease in transcription of genes related to SPS in the *Enterobacter* treatment 9 dpi can explain why this bacterium was not able to confer better drought tolerance compared to the *Bacillus* treatment. In the *Bacillus* treatment, even if the SPS genes expressed 5 dpi were downregulated; two out of the three genes expressed 9 dpi were upregulated. The upregulation of genes related to SPS was also reported by Skz et al. (2018) during his study on maize seedlings inoculated with *P. putida*. Also, the beneficial action of *Bacillus subtilis* on timothy grass and *Neotyphodium coenophialum* in tall fescue plants were linked to the accumulation of sucrose (Nagabhyru et al., 2013, Gagné-Bourque et al., 2016).

In our study, a gene involved in the starch break down, Beta amylase, was highly upregulated and present only in the *Bacillus* treatment 5 dpi. This result is similar to the one obtained by Skz et al. (2018). The stored starch breakdown into small oligosaccharides provides energy to the plants under drought stress (Kaplan & Guy, 2004). Our results also showed the upregulation of a gene involved in starch synthesis, GBSSIIa, only present in the *Bacillus* treatment 9 dpi. The study by Zheng et al. (2020) reported 37 genes linked to the tolerance of maize to drought, which was associated with the starch synthesis were upregulated. All these results together suggest that the *Bacillus* treatment increases the activity of starch responsive genes that will allow the plant to continue growing despite

the drought. The absence of starch in the *Enterobacter* treatment can explain its inability to confer drought tolerance as efficiently as the *Bacillus* treatment.

DEGs related to plant-pathogen interaction

According to Frolov et al. (2017), upon exposure to abiotic stress, the first step to ensure plant survival is the signal perception linked to this stress. The abiotic stress is first perceived by the receptors present on cell membranes and transduced downstream resulting in the generation of secondary messengers including K^+, Ca^{2+}, sugars, ROS, cyclic nucleotides, and inositol phosphates. The calcium ion (Ca^{2+}) is recognized as an important secondary messenger in plants. Its signals are surmised to regulate most aspects of plant growth and development, including response to drought (Jiang et al., 2013, Kong et al., 2013). Besides that, these ion signals are associated with many responses to plant hormones, including Abscisic Acid, Gibberellic Acid, Cytokinin, Auxin, Brassinolides, Jasmonic Acid, and Ethylene.

The calcium-dependent protein kinases (CDPKs) are vitally involved in plant abiotic stress responses. For example, the overexpression of a *CDPK* gene in rice *(OsCDPK7)* was able to alleviate both cold and salt/drought tolerance in rice plants and its suppression lowered stress tolerance (Saijo et al., 2000). In our study, five genes were linked to CDPKs. Five days post-inoculation, 2 of these genes were identified in the *Bacillus* treatment and one in the *Enterobacter* treatment. Between the two genes present in the *Bacillus* treatment, one was downregulated and the second upregulated. Nine dpi, these signal genes were not present in the *Enterobacter* treatment. In the *Bacillus* treatment, there were three genes regulated, one highly upregulated and the two others

lowly downregulated. These results suggest that the beneficial action of the *Enterobacter* can be linked to the early action of the CDPKs genes, but the activity of this gene is controlled in the *Bacillus* treatment in order to maintain its ideal activity balance. In the same topic, 4 genes related to the calmodulin-like proteins (CMLs) major Ca^{2+} sensors, were found in the *Bacillus* treatment and were all downregulated suggesting that the beneficial action of the *Bacillus* is not linked to Ca^{2+} activity.

A study by Weidenbach et al. (2015) reported that a barley mutant with a defect in the *3-KETOACYL-CoA-SYNTHASE* gene resulted in reduced amounts of cuticular waxes on plant cells. As a consequence of this mutation, under drought stress conditions, enhanced lateral root development was recorded. In our study, three genes were found related to 3-ketoacyl-CoA synthase. Five days post-inoculation; this gene was only present in the *Bacillus* treatment and was upregulated. This suggests that it allowed the plants to produce more roots in order to use water more efficiently to continue growing and storing more water in the aerial parts. Nine days post-inoculation, two genes were expressed by the two bacteria related to 3-ketoacyl-CoA synthase, one common to both, and the other only expressed in the *Bacillus* treatment. Both genes were downregulated suggesting that under advanced drought conditions, the two bacteria are repressing the activity of genes related to root proliferation in order to save energy for the plant and this repression being high in the *Bacillus* treatment may explain why this bacterium conferred better drought tolerance.

According to Wang et al. (2018), the WRKY family gene is involved in plant responses to abiotic stresses. As an example, the overexpression of the WRKY30 in rice

led to an enhanced resistance of this plant to drought stress. In our study, two WRKY genes were found, the WRKY33 and the WRKY2. WRKY33, reported as able to confer salt tolerance to *Arabidopsis* (Li et al., 2013), was downregulated and only present in the *Bacillus* treatment 9 dpi. In *Arabidopsis*, the WRKY2 TF, is reported to mediate seed germination and postgermination developmental arrest by ABA (Jiang & Yu, 2009). In our study, the WRKY2 TF was also downregulated and only present in the *Enterobacter* treatment 9 dpi. This decreased in the WRKY 2 transcript suggests that the *Enterobacter* treatment 9 dpi allowed the plant to continue growing despite drought

Heat shock proteins (HSP) because of being associated with protein folding, localization, accumulation, and degradation are surmised to play a crucial role in many cellular processes and to aid in abiotic stress tolerance (Wang et al., 2004). In our study, one HSP was upregulated and was only expressed in the *Bacillus* treatment 9 dpi. These results are consistent with those reported by Skz et al. (2018) working on maize and Lim and Kim (2013) working on pepper, that the beneficial effect of PGPRs was linked to the accumulation of HSP. The non-expression of the HSP by the *Enterobacter* treatments can also be linked to their inability to confer drought alleviation as much as the *Bacillus* treatment.

DEGs linked to the Phenylpropanoid biosynthesis pathway

Under abiotic stress conditions like drought, the Phenylpropanoid biosynthetic pathway is activated and as a result, and there is an accumulation of various phenolic compounds which, among other roles, have the potential to scavenge harmful reactive oxygen species (ROS) (Sharma et al., 2019). The majority of genes obtained during our

study and linked to the Phenylpropanoid biosynthetic pathway were associated with peroxidase activity (23 in 34). Peroxidases are enzyme with two functions: in the standard peroxidative cycle, they can oxidize various substrates in the presence of H_2O_2 (a well-known ROS), but can also produce ROS in their oxidative cycles acting therefore as pro- or anti-oxidant enzymes (Kravić et al., 2013). Five days post-inoculation, three downregulated genes linked to peroxidase activity, were found and only in the *Bacillus* treatment. Nine days post-inoculation, in the *Bacillus* treatment, there were 17 DEGs all downregulated related to the peroxidase activity while in the *Enterobacter* treatment, there were 11 genes with two upregulated and nine downregulated.

Beta Glucosidases are enzymes associated with various biological processes, such as defense against pests, phytohormone activation, lignification, and cell wall catabolism. Beta-glucosidase transcripts were shown to be induced by saline stress and water stress in rice and *Arabidopsis thaliana* (Riccardi et al., 2004). In our study, three genes were linked to the Beta glucosidase pathway and were downregulated and were only found 9 dpi in the two bacterial treatments. The downregulation in our study suggests that the two bacteria are reducing the activity of genes associated with root's lignification in order to allow the plant to take up more water and reducing cell wall catabolism to preserve cell integrity.

In conclusion to our results,

In our study, based on the results obtained show that,

- The results obtained show that there was more water retained in seedlings leaves inoculated with the *Bacillus* 12D6 strain than the *Enterobacter* 16i strain compared to plants under drought without inoculation.
- There were differences in terms of roots lengths and the number of tips produced between the inoculated seedlings and those under drought.
- The expression of genes linked to the JA pathway was high in the early stage of the drought in seedlings inoculated with the *Bacillus* compared to the advanced days of the drought. This early expression of genes associated with the JA suggests that the beneficial action of the *Bacillus* can be linked to the JA pathway. The expression of genes linked to the JA pathway was decreased in the *Enterobacter* treatments.
- Genes linked to the Ethylene pathway were downregulated in the *Bacillus* treatment, but these genes were not differentially expressed in the *Enterobacter* treatment.
- The expression of Auxin-responsive genes was induced by the two bacteria 5 dpi but repressed 9 dpi.
- There was an overall downregulation of genes encoding for the TPP in the two bacteria at both time points.
- The expression of ABA-responsive genes was decreased in the plants inoculated with the two bacteria 9 dpi, but no gene linked to ABA was differentially expressed 5 dpi in the two bacterial treatments.

- A downregulation of genes related to the TGA transcription factors was observed 9 dpi in the two bacteria, but these genes were not differentially expressed 5 dpi.
- A majority of genes related to trehalose production was downregulated in the two bacteria during both time points, but one gene was found upregulated in the *Enterobacter* treatment 9 dpi.
- A decrease in transcripts abundance of DEGs related to beta-glucosidase production was observed 9 dpi in both bacteria
- The expression of genes related to sucrose was decreased in the *Enterobacter* treatment, but increased in the *Bacillus* treatment and observed only 9 dpi.
- The *Bacillus* treatment has increased the expression of starch genes while a decrease in starch responsive genes was observed in the *Enterobacter* treatment.
- The activity of genes related to the ion $Ca^{2+}$was enhanced in the *Enterobacter* treatment 5 dpi.
- The expression of genes associated with the *3-KETOACYL-CoA-SYNTHASE* was increase, 5 dpi in both bacteria, and repressed 9 dpi.
- There was a decrease in the WRKY 2 transcript abundance in the *Enterobacter* treatment 9 dpi not observed in the *Bacillus* treatment which was not differentially expressed.
- An accumulation of genes linked to HSP was observed in plants inoculated with the *Bacillus* but these genes were not expressed in the *Enterobacter* treatments
- The genes related to the peroxidase activity were repressed in the *Bacillus* treatment during both time points. In the *Enterobacter* treatment, there was no

peroxidase activity 5 dpi; and at 9 dpi, between the genes involved, there were two upregulated.

- The activity of genes associated with Beta glucosidase production was reduced and present in the two bacteria 9 dpi.

CHAPTER V

CONCLUSION

In summary of our study, we were able to show the ability of the two bacteria, *Bacillus* 12D6 and *Enterobacter* 16i, to induce drought tolerance in maize seedlings by helping plants retain more water and alter the root system architecture. The study of the root transcriptomes 5 and 9 days post-inoculation using RNA sequencing have allowed for the detection of the major genes induced or repressed by the two bacterial strains added to the soil function in hormone transduction, phenylpropanoid biosynthesis, starch, and sucrose metabolism, and plant-pathogen interaction. We also discussed the potential role of these genes in the drought alleviation in maize seedlings. Our results show both different and common patterns on the regulation of genes linked to ethylene biosynthesis, abscisic acid and auxin signaling, superoxide dismutase, catalase, and peroxidase, heat shock proteins, and late embryogenesis abundant in the seedlings inoculated with the two bacteria. In addition to our study and in order to facilitate the implementation of PGPRs in fields, more studies like the effect of the combination of the two bacteria on plant phenotype, the effect of different types of soil on the two bacteria performance … are needed in order to gain the full knowledge of the beneficial actions linked to these bacteria.

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APPENDIX A

	Total reads		
Treatments	**Read 1**	**Read 2**	**Total reads**
T4_1	15,552,587	15,552,585	31,105,172
T4_2	13,298,179	13,298,180	26,596,359
T4_3	14,760,154	14,760,152	29,520,306
12D6_1_TP2	14,971,501	14,971,502	29,943,003
12D6_2_TP2	14,140,245	14,140,245	28,280,490
12D6_3_TP2	15,447,665	15,447,664	30,895,329
12D6_1_TP3	16,252,598	16,252,598	32,505,196
12D6_2_TP3	16,531,796	16,531,794	33,063,590
12D6_3_TP3	16,227,341	16,227,343	32,454,684
16i_1_TP2	15,959,070	15,959,069	31,918,139
16i_2_TP2	15,000,969	15,000,971	30,001,940
16i_3_TP2	18,122,151	18,122,152	36,244,303
16i_1_TP3	15,743,443	15,743,444	31,486,887
16i_2_TP3	16,048,320	16,048,321	32,096,641
16i_3_TP3	14,245,792	14,245,793	28,491,585
W_neg_1_TP2	14,330,849	14,330,850	28,661,699
W_neg_2_TP2	14,973,316	14,973,317	29,946,633
W_neg_3_TP2	14,887,977	14,887,979	29,775,956
W_neg_1_TP3	15,643,011	15,643,012	31,286,023
W_neg_2_TP3	14,921,421	14,921,422	29,842,843
W_neg_3_TP3	15,939,968	15,939,969	31,879,937
W_pos_1_TP2	15,944,359	15,944,359	31,888,718
W_pos_2_TP2	15,930,802	15,930,801	31,861,603
W_pos_3_TP2	15,034,254	15,034,253	30,068,507
W_pos_1_TP3	18,758,157	18,758,157	37,516,314
W_pos_2_TP3	14,758,064	14,758,062	29,516,126
W_pos_3_TP3	15,865,977	15,865,975	31,731,952
Total	419,289,966	419,289,969	838,579,935

APPENDIX B

G.O. category Biological Process:

High level GO category	Number of genes in list							
	12D6				16i			
	5 dpi		9 dpi		5 dpi		9 dpi	
Response to stimulus	60	11%	316	8%	26	9%	144	8%
Regulation of metabolic process	35	6%	302	7%	14	5%	127	7%
Localization	27	5%	286	7%	21	7%	142	7%
Establishment of localization	27	5%	276	7%	21	7%	137	7%
Cellular component organization or biogenesis	15	3%	241	6%	7	2%	93	5%
Cellular response to stimulus	43	8%	220	5%	17	6%	95	5%
Catabolic process	39	7%	202	5%	15	5%	89	5%
Cellular component organization	15	3%	191	5%	7	2%	81	4%
Response to stress	38	7%	159	4%	12	4%	56	3%
Response to chemical	36	6%	151	4%	14	5%	73	4%
Cellular component biogenesis	6	1%	117	3%	3	1%	35	2%
Signaling	21	4%	116	3%	9	3%	61	3%
Developmental process	13	2%	102	2%	8	3%	52	3%
Macromolecule localization	3	1%	82	2%	3	1%	33	2%
Multicellular organismal process	10	2%	78	2%	4	1%	43	2%
Regulation of biological quality	10	2%	76	2%	8	3%	40	2%
Cellular localization	3	1%	73	2%	3	1%	24	1%
Positive regulation of biological process	6	1%	70	2%	7	2%	27	1%
Anatomical structure development	10	2%	69	2%	5	2%	44	2%
Reproduction	4	1%	63	2%	2	1%	33	2%
Reproductive process	4	1%	63	2%	2	1%	33	2%
Response to endogenous stimulus	18	3%	61	1%	8	3%	35	2%
Negative regulation of biological process	4	1%	59	1%	2	1%	24	1%
Multi-organism process	9	2%	56	1%	4	1%	22	1%
Methylation	6	1%	55	1%	4	1%	16	1%
Cell wall organization or biogenesis	5	1%	55	1%	5	2%	48	3%
Regulation of molecular function	7	1%	54	1%	3	1%	22	1%
Response to abiotic stimulus	12	2%	47	1%	4	1%	28	1%
Response to external stimulus	6	1%	41	1%	4	1%	14	1%
Detoxification	6	1%	41	1%	2	1%	16	1%
Cellular detoxification	5	1%	39	1%	2	1%	15	1%

Cell cycle process	0	0%	29	1%	0	0%	18	1%
Developmental process involved in reproduction	2	0%	28	1%	2	1%	18	1%
Regulation of response to stimulus	7	1%	28	1%	4	1%	13	1%
Response to biotic stimulus	6	1%	26	1%	4	1%	10	1%
Hormone metabolic process	5	1%	24	1%	3	1%	14	1%
Response to other organism	6	1%	22	1%	4	1%	7	0%
Anatomical structure morphogenesis	4	1%	19	0%	0	0%	11	1%
Regulation of developmental process	3	1%	18	0%	5	2%	11	1%
Protein folding	6	1%	17	0%	0	0%	4	0%
Multi-multicellular organism process	2	0%	16	0%	0	0%	5	0%
Regulation of multicellular organismal process	2	0%	15	0%	4	1%	7	0%
Sexual reproduction	0	0%	14	0%	0	0%	9	0%
Multi-organism reproductive process	0	0%	14	0%	0	0%	9	0%
Growth	0	0%	13	0%	0	0%	6	0%
Autophagy	0	0%	12	0%	0	0%	2	0%
Regulation of reproductive process	0	0%	11	0%	2	1%	6	0%
Regulation of signaling	5	1%	10	0%	0	0%	7	0%
Immune system process	3	1%	9	0%	2	1%	3	0%
Immune response	3	1%	9	0%	2	1%	3	0%
Meiotic cell cycle process	0	0%	9	0%	0	0%	6	0%
Developmental growth	0	0%	8	0%	0	0%	5	0%
Regulation of cellular component biogenesis	0	0%	7	0%	0	0%	3	0%
Anatomical structure formation involved in morphogenesis	0	0%	7	0%	0	0%	3	0%
Cell growth	0	0%	6	0%	0	0%	4	0%
Maintenance of location	0	0%	6	0%	2	1%	2	0%
Activation of immune response	0	0%	5	0%	0	0%	0	0%
Regulation of immune system process	0	0%	5	0%	2	1%	0	0%
Regulation of localization	5	1%	5	0%	2	1%	3	0%
Regulation of growth	0	0%	5	0%	0	0%	2	0%
Interspecies interaction between organisms	0	0%	5	0%	0	0%	0	0%
Cell proliferation	0	0%	3	0%	0	0%	2	0%
Immune effector process	0	0%	2	0%	0	0%	0	0%
Pathogenesis	0	0%	2	0%	0	0%	0	0%
Intercellular transport	0	0%	2	0%	0	0%	0	0%
Locomotion	0	0%	2	0%	0	0%	0	0%
Regulation of multi-organism process	0	0%	2	0%	0	0%	0	0%
Detection of stimulus	2	0%	2	0%	2	1%	0	0%

Multicellular organism reproduction	0	0%	0	0%	0	0%	3	0%
Multicellular organismal reproductive process	0	0%	0	0%	0	0%	3	0%
Carbon utilization	0	0%	0	0%	0	0%	2	0%

G.O. category Cellular Component:

High level GO category	Number of genes in list							
	12D6				16i			
	5 dpi		9 dpi		5 dpi		9 dpi	
Cell periphery	25	15%	336	12%	19	13%	191	17%
Organelle part	19	11%	311	11%	14	10%	102	9%
Intracellular organelle part	19	11%	311	11%	14	10%	102	9%
Plasma membrane	18	11%	257	9%	13	9%	149	13%
Protein-containing complex	11	6%	240	8%	6	4%	57	5%
Non-membrane-bounded organelle	3	2%	148	5%	5	4%	39	3%
Endomembrane system	9	5%	128	4%	8	6%	67	6%
Extracellular region	15	9%	109	4%	9	6%	43	4%
Membrane-enclosed lumen	4	2%	105	4%	0	0%	10	1%
Organelle lumen	4	2%	105	4%	0	0%	10	1%
Ribonucleoprotein complex	0	0%	99	3%	0	0%	6	1%
External encapsulating structure	7	4%	75	3%	6	4%	38	3%
Cell-cell junction	4	2%	72	2%	5	4%	44	4%
Cell junction	4	2%	72	2%	5	4%	44	4%
Symplast	4	2%	72	2%	5	4%	44	4%
Organelle membrane	8	5%	60	2%	6	4%	26	2%
Plasma membrane part	4	2%	60	2%	3	2%	41	4%
Envelope	0	0%	39	1%	0	0%	12	1%
Whole membrane	7	4%	33	1%	6	4%	19	2%
Membrane protein complex	3	2%	26	1%	0	0%	6	1%
Endoplasmic reticulum membrane	0	0%	23	1%	3	2%	9	1%
Nuclear outer membrane-endoplasmic reticulum membrane network	0	0%	23	1%	3	2%	9	1%
Apoplast	3	2%	16	1%	2	1%	9	1%
Supramolecular fiber	0	0%	15	1%	0	0%	17	1%

Polymeric cytoskeletal fiber	0	0%	15	1%	0	0%	17	1%
Chromatin	0	0%	14	0%	3	2%	3	0%
Protein-DNA complex	0	0%	13	0%	3	2%	3	0%
Outer membrane	0	0%	12	0%	0	0%	3	0%
Mitochondrial membrane part	0	0%	11	0%	0	0%	0	0%
Extracellular space	0	0%	10	0%	2	1%	2	0%
Extracellular region part	0	0%	10	0%	2	1%	2	0%
Virion	0	0%	8	0%	0	0%	0	0%
Viral nucleocapsid	0	0%	8	0%	0	0%	0	0%
Viral capsid	0	0%	8	0%	0	0%	0	0%
Photosynthetic membrane	0	0%	8	0%	0	0%	4	0%
Virion part	0	0%	8	0%	0	0%	0	0%
Extrinsic component of membrane	0	0%	7	0%	0	0%	2	0%
Intrinsic component of organelle membrane	0	0%	7	0%	0	0%	0	0%
Oxidoreductase complex	0	0%	7	0%	0	0%	3	0%
Nucleoid	0	0%	5	0%	0	0%	0	0%
Organellar ribosome	0	0%	4	0%	0	0%	0	0%
Methyltransferase complex	0	0%	4	0%	0	0%	0	0%
Cell leading edge	0	0%	3	0%	0	0%	0	0%
Mitochondrial nucleoid	0	0%	3	0%	0	0%	0	0%
Cell projection	0	0%	3	0%	0	0%	0	0%
Cell projection part	0	0%	3	0%	0	0%	0	0%
Coated membrane	0	0%	3	0%	0	0%	0	0%
Plasma membrane region	0	0%	3	0%	0	0%	0	0%
Cell division site	0	0%	2	0%	0	0%	0	0%
Cell division site part	0	0%	2	0%	0	0%	0	0%
Plastid nucleoid	0	0%	2	0%	0	0%	0	0%
Clathrin-coated pit	0	0%	0	0%	0	0%	2	0%

G.O. category Molecular function:

High level GO category	**Number of genes in list**							
	12D6				**16i**			
	5 dpi		**9 dpi**		**5 dpi**		**9 dpi**	
Small molecule binding	41	14%	39	16%	18	15%	17	15%
Carbohydrate derivative binding	30	10%	34	14%	15	12%	15	13%
Drug binding	29	10%	32	13%	13	11%	14	12%

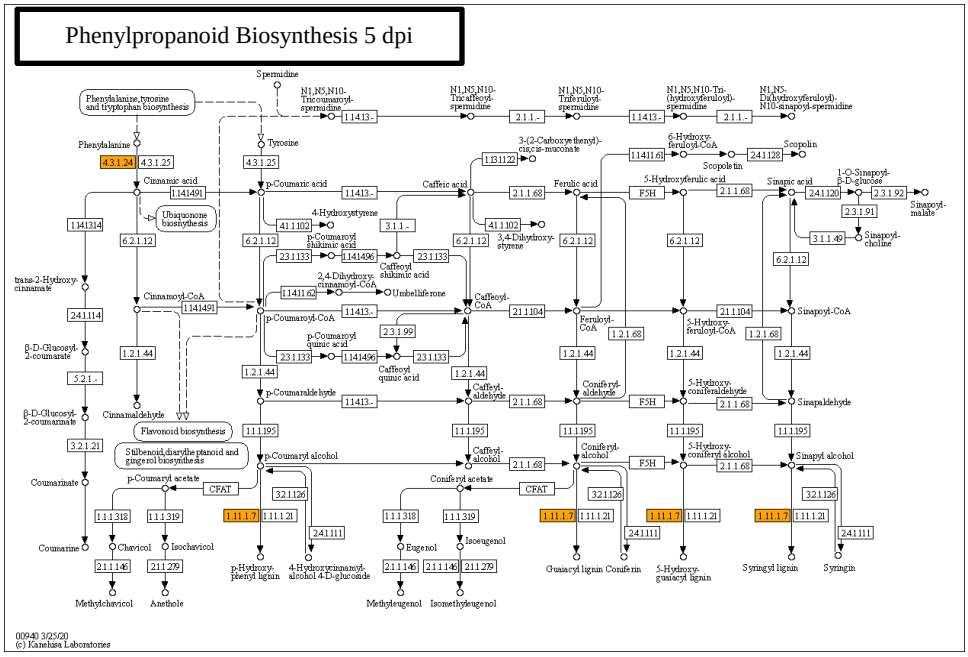
Oxidoreductase activity	37	13%	212	9%	16	13%	121	10%
Transporter activity	19	7%	169	7%	18	15%	94	8%
Transmembrane transporter activity	19	7%	155	6%	16	13%	89	8%
Cofactor binding	34	12%	149	6%	10	8%	82	7%
DNA-binding transcription factor activity	18	6%	125	5%	5	4%	71	6%
Structural molecule activity	2	1%	48	2%	0	0%	5	0%
Ligase activity	4	1%	43	2%	2	2%	14	1%
Carbohydrate binding	6	2%	40	2%	2	2%	17	1%
Molecular transducer activity	2	1%	40	2%	0	0%	24	2%
Lyase activity	8	3%	38	2%	2	2%	14	1%
Molecular function regulator	5	2%	38	2%	0	0%	17	1%
Structural constituent of ribosome	0	0%	37	1%	0	0%	3	0%
Antioxidant activity	5	2%	37	1%	2	2%	15	1%
Enzyme regulator activity	5	2%	37	1%	0	0%	13	1%
Signaling receptor activity	0	0%	35	1%	0	0%	20	2%
Peroxidase activity	5	2%	34	1%	2	2%	14	1%
Isomerase activity	2	1%	29	1%	0	0%	8	1%
Lipid binding	0	0%	25	1%	0	0%	19	2%
Nutrient reservoir activity	0	0%	22	1%	0	0%	0	0%
Electron transfer activity	2	1%	19	1%	0	0%	18	2%
Protein-containing complex binding	0	0%	19	1%	0	0%	3	0%
Amide binding	2	1%	12	0%	0	0%	7	1%
Pattern binding	3	1%	10	0%	0	0%	5	0%
Sulfur compound binding	2	1%	9	0%	0	0%	8	1%
Modified amino acid binding	0	0%	8	0%	0	0%	7	1%
Hormone binding	0	0%	7	0%	0	0%	2	0%
Structural constituent of nuclear pore	0	0%	6	0%	0	0%	0	0%
Metal cluster binding	2	1%	5	0%	0	0%	2	0%
Structural constituent of cytoskeleton	2	1%	4	0%	0	0%	0	0%
Cyclase activity	0	0%	3	0%	0	0%	0	0%
Deaminase activity	0	0%	3	0%	0	0%	0	0%
Thioredoxin-disulfide reductase activity	0	0%	2	0%	0	0%	0	0%
Demethylase activity	0	0%	2	0%	0	0%	2	0%
Carbohydrate derivative transmembrane transporter activity	0	0%	2	0%	0	0%	2	0%
Fatty acid derivative binding	2	1%	0	0%	0	0%	0	0%
Guanyl-nucleotide exchange factor activity	0	0%	0	0%	0	0%	4	0%
Demethylase activity	0	0%	0	0%	0	0%	2	0%

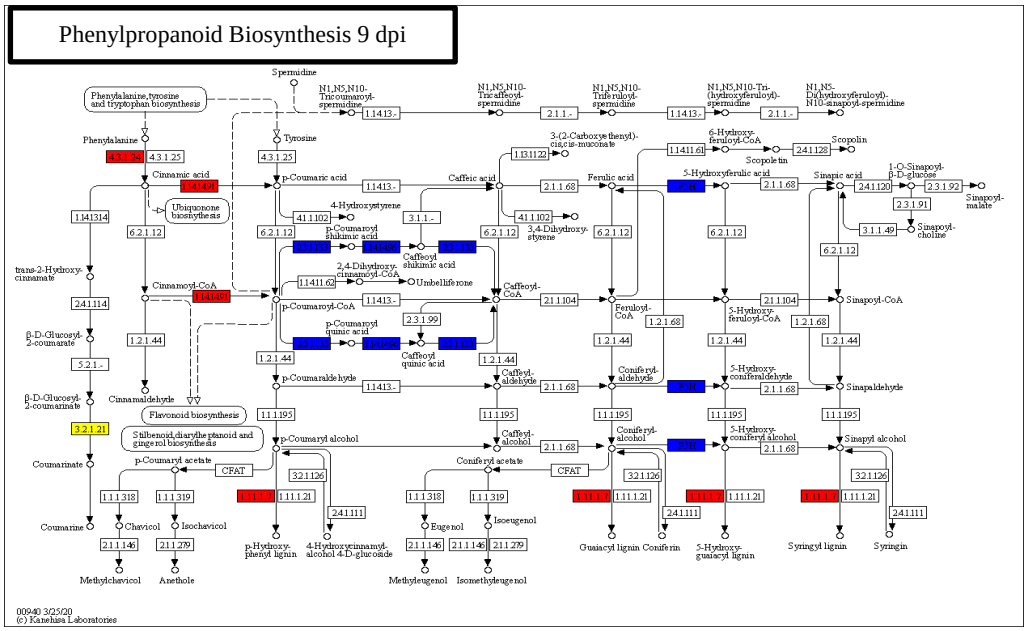
Carbohydrate derivative transmembrane transporter activity	0	0%	0	0%	0	0%	2	0%

APPENDIX C

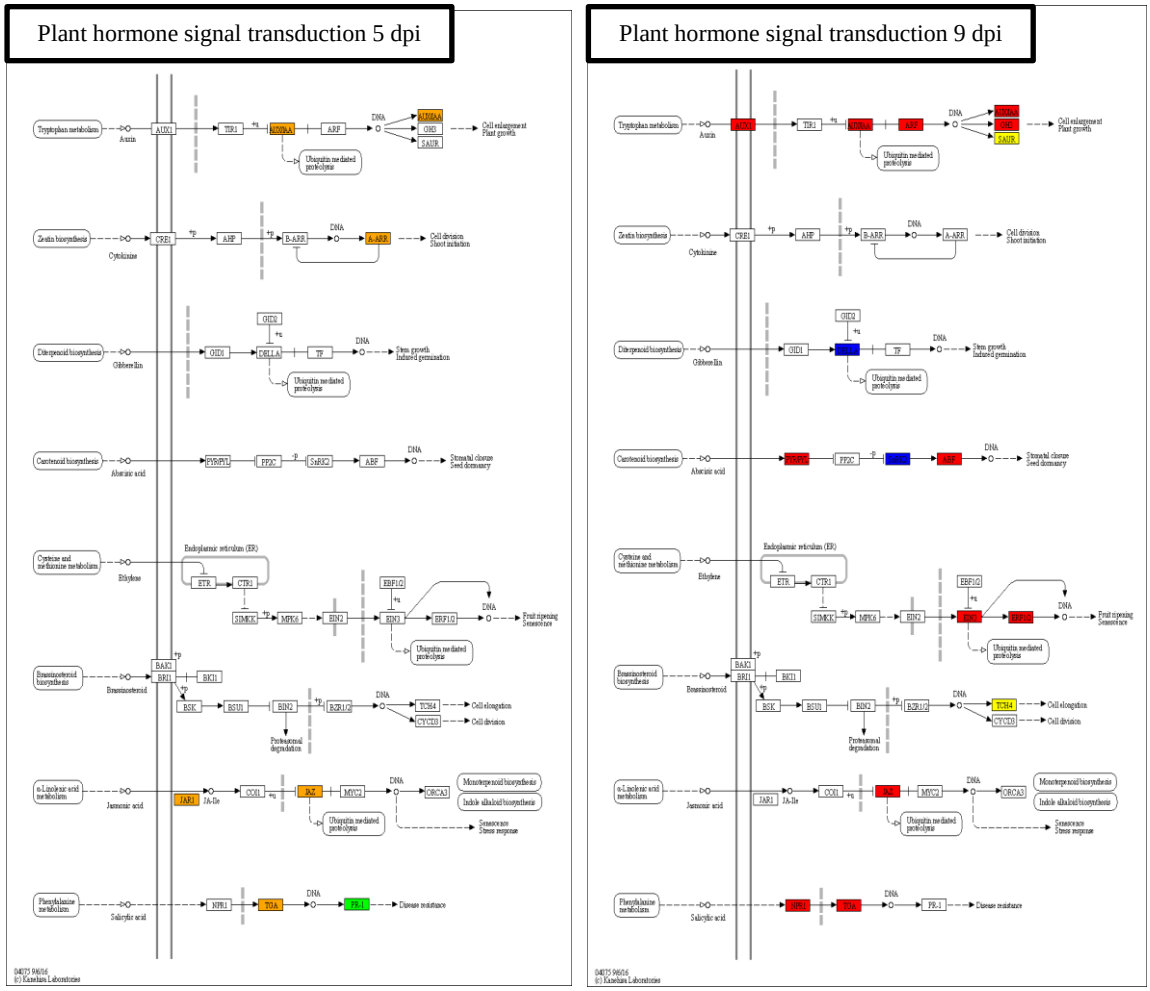
16i_TP2	green
12D6_TP2	orange
Common 16i & 12D6_TP2	purple

16i_TP3	blue
12D6_TP3	red
Common 16i & 12D6_TP3	yellow



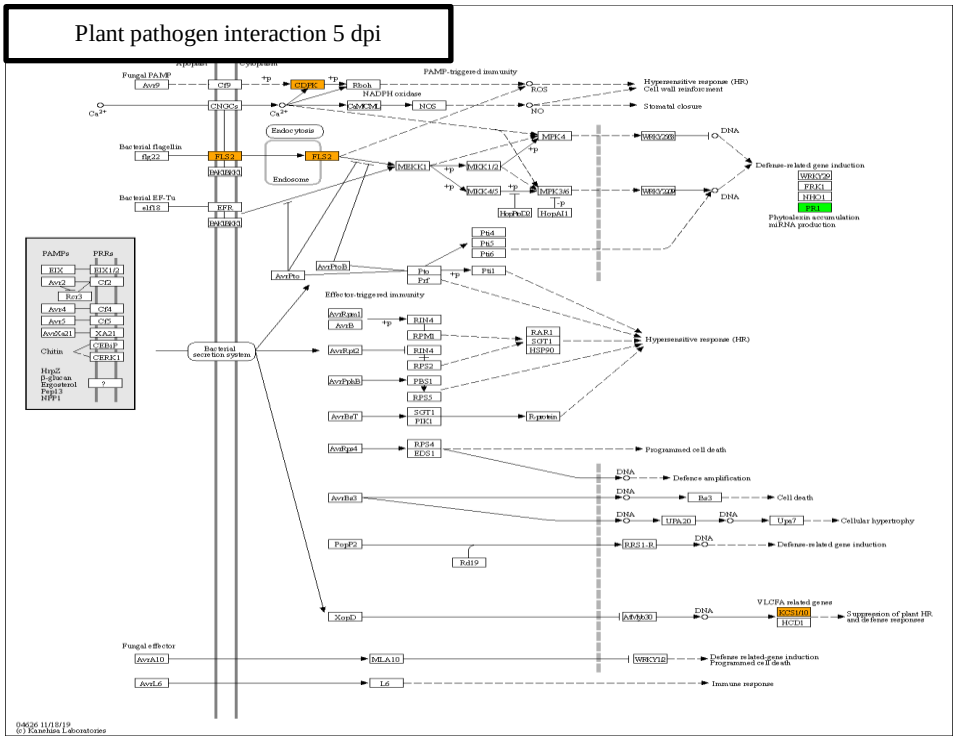


C1: KEGG map of Phenylpropanoid Biosynthesis

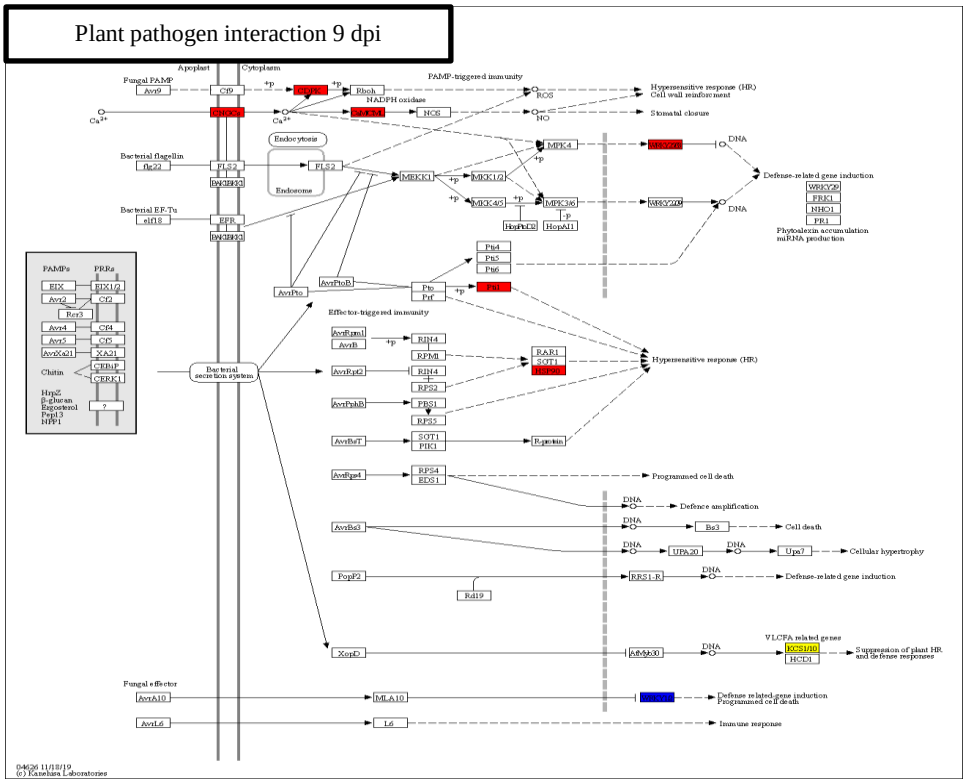


C2: KEGG map of Plant hormone signal transduction

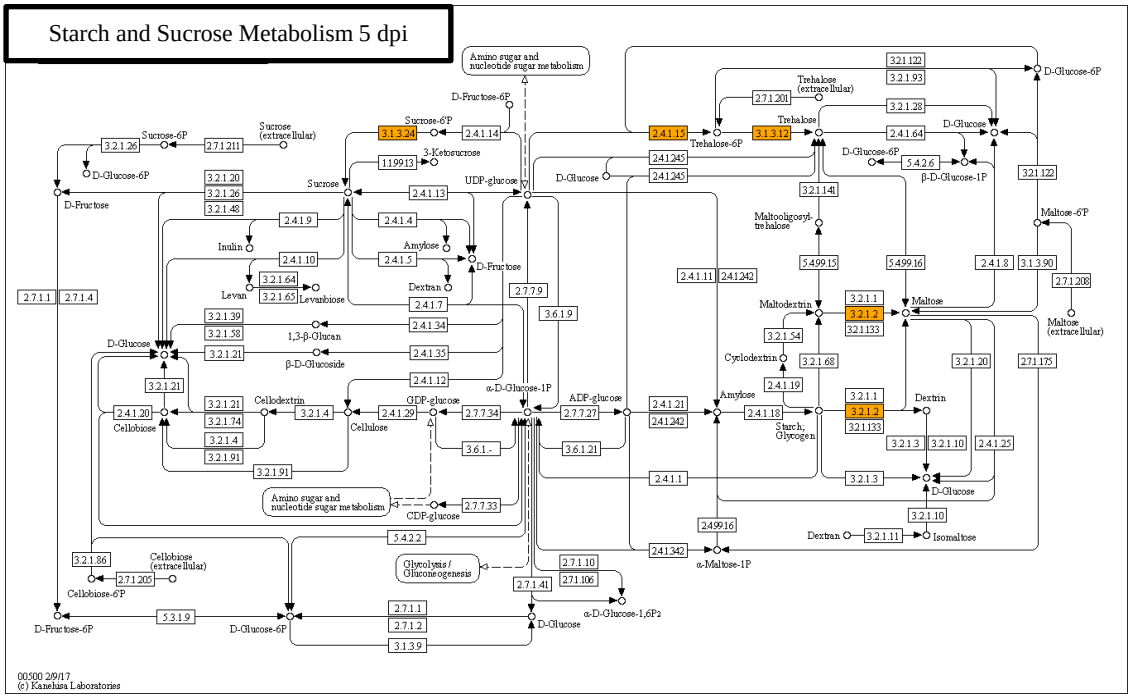
Plant pathogen interaction 5 dpi

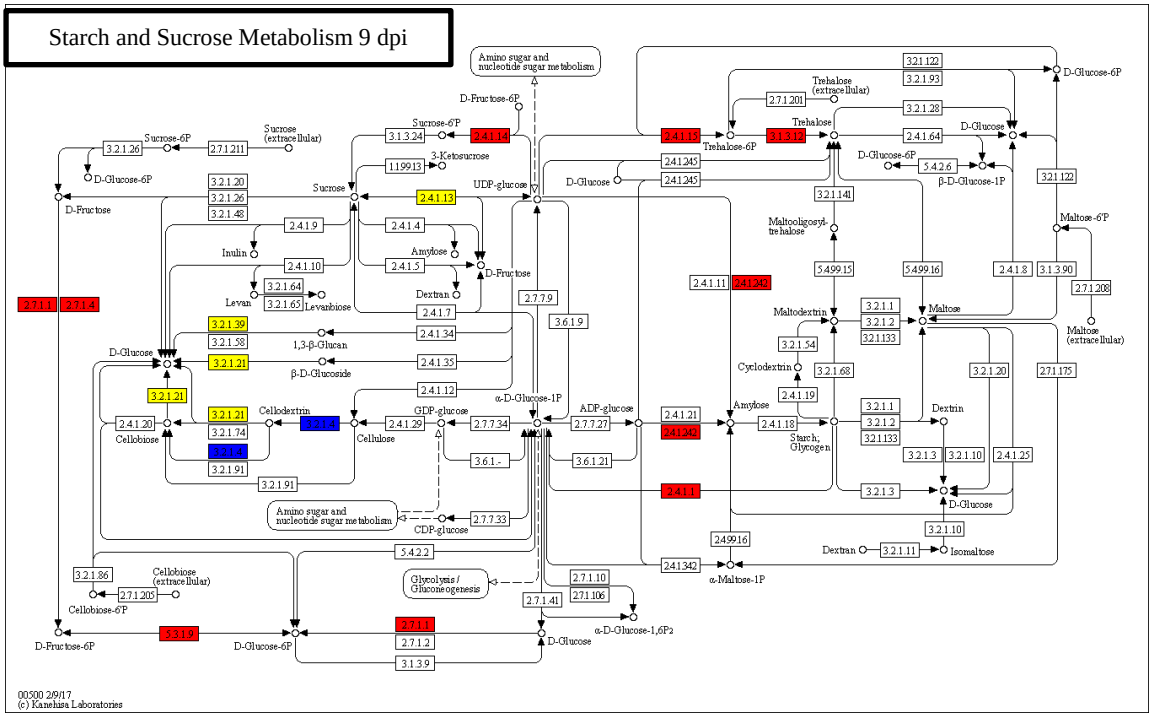


Plant pathogen interaction 9 dpi



C3: KEGG map of Plant pathogen interaction





KEGG map of Starch and Sucrose Metabolism.