The Genetic Regulation of Secondary Metabolic Pathways in Response to Salinity and Drought as Abiotic Stresses

Sameer Hasan Qari 1,2,* and Ibrahim Tarbiyyah 1,2,*

1 Biology Department, Aljumum University College, Umm Al-Qura University, Makkah 24224, Saudi Arabia
2 Genetics and Molecular Biology Central Laboratory (GMCL), Aljumum University College, Umm Al-Qura University, Makkah 24224, Saudi Arabia
* Correspondence: shqari@uqu.edu.sa (S.H.Q.); s44181979@st.uqu.edu.sa (I.T.)

Abstract: Global development has generated a plethora of unfavorable and adverse environmental factors for the living organisms in the ecosystem. Plants are sessile organisms, and they are crucial to sustain life on earth. Since plants are sessile, they face a great number of environmental challenges related to abiotic stresses, such as temperature fluctuation, drought, salinity, flood and metal contamination. Salinity and drought are considered major abiotic stresses that negatively affect the plants’ growth and production of useful content. However, plants have evolved various molecular mechanisms to increase their tolerance to these environmental stresses. There is a whole complex system of communication (cross-talk) through massive signaling cascades that are activated and modulated in response to salinity and drought. Secondary metabolites are believed to play significant roles in the plant’s response and resistance to salinity and drought stress. Until recently, attempts to unravel the biosynthetic pathways were limited mainly due to the inadequate plant genomics resources. However, recent advancements in generating high-throughput “omics” datasets, computational tools and functional genomics approach integration have aided in the elucidation of biosynthetic pathways of many plant bioactive metabolites. This review gathers comprehensive knowledge of plants’ complex system that is involved in the response and resistance to salinity and water deficit stresses as abiotic stress. Additionally, it offers clues in determining the genes involved in this complex and measures its activity. It covers basic information regarding the signaling molecules involved in salinity and drought resistance and how plant hormones regulate the cross-talking mechanism with emphasis on transcriptional activity. Moreover, it discusses many studies that illustrate the relationship between salinity and drought and secondary metabolite production. Furthermore, several transcriptome analysis research papers of medicinal plants are illustrated. The aim of this review is to be a key for any researcher that is aspiring to study the relationship between salinity and drought stresses and secondary metabolite production at the transcriptome and transcription level.

Keywords: salinity; drought; abiotic stress; phytohormones; ROS; signal transduction; cross-talk; secondary metabolite; transcriptome analysis

1. Introduction

Due to the plants’ sessility and inability to migrate in nature, they are more prone to being affected by a great number of abiotic stresses such as salinity, drought, heat and radiation. Those abiotic stresses are considered major challenges for the development and growth of the plants and potentially influence the yield and quality of the useful products that provide medicinal or nutritional benefits to humans [1]. Hence, understanding the mechanisms by which plants react to abiotic stresses will aid in improving the quality and quantity of the rich and diverse natural products.

Many of these stressors induce elicitors that would eventually result in either negative or positive plant responses [2]. For instance, a positive influence has been proved by Crisosto [3] in a study on peaches; it showed that a higher fruit density was observed at
lower levels of irrigation. On the contrary, abiotic stresses also have negative effects on the development and growth of the plant. For example, they can result in changing the reactive oxygen species (ROS) levels in the body and cause damage; these changes will force the plant to undergo programmed cell death (PCD) [4]. Other examples of negative effects of abiotic stresses are the osmotic and ionic stresses that occur when plants are exposed to increased levels of salinity and water shortage [5].

The plants tend to adapt to those abiotic stresses in order to survive and thrive [6]. There are many players that contribute to the process of resistance, including signaling molecules, DNA-binding proteins and an enormous number of stress-inducible genes. Those biological players participate in the complex orchestration of the plants’ tolerance to stress at the molecular level. Stress-related molecules such as abscisic acid (ABA), jasmonic acid (JA) [7], ROS, calcium ions, a variety of transcription factors (TFs) and other regulatory and functional proteins all interact together in a spectacular manner in order to perform a remarkable adaptation to the abiotic stresses [8,9]. This communication between secondary messengers, phytohormones, DNA-binding proteins and stress-inducible genes is known as “cross-talk” [9,10].

In response to abiotic stresses, such as salinity and drought, the genetic regulation also involves a complex organization of metabolic activities that involve the intracellular production of beneficial secondary metabolites. Those secondary metabolites facilitate the plant’s defense mechanism by conferring many biological activities such as protecting the plant against oxidative stress, and they are known as stress-protectant metabolites [11,12]. A number of studies have undisputedly determined that many secondary metabolites such as flavonoids, sesquiterpenes and phenolic acids have a substantial antioxidant activity role. Since the changing balance of the ROS in the plant occurs when plants are subjected to abiotic stress, these antioxidative secondary metabolites contribute to the defense mechanisms that the plant utilizes to overcome the harmful effects of stress [13–15]. Many plant-based secondary metabolites provide beneficial uses as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides and food additives [16,17]. There are many secondary metabolites with beneficial use as a dietary component or for medicinal purposes, including flavonoids (anthocyanins), alkaloids, terpenes and many others [11,12].

Throughout history, genetic manipulation techniques led to the improvement of the plant’s production and quality of useful compounds and resistance to abiotic stresses. Plant natural products have been considered essential phytochemicals that have been used extensively in the field of medicine throughout human history. Some of the well-known plant-extract-derived medicines are morphine, quinine, colchicine, codeine and many others [18]. The anticancer drug that is known as Taxol is extracted from trees from the Taxus genus [12]. A remarkable number of studies on medicinal plants displayed crucial evidence that when they are subjected to abiotic stress, the accumulation of secondary metabolites is usually achieved [19–22]. As a result of these findings, the interest in plants’ defense mechanisms and their secondary metabolite production has expanded and been focused on by many researchers. This brief review summarizes the influence of different abiotic factors including salt and drought on secondary metabolite production in plants. The focus of the present review is on the regulatory mechanisms by which plants enhance their resistance to salinity and drought involving several players including secondary messengers, phytohormones, transcription factors and stress-inducible genes. This review also focuses on how these regulatory events impact the secondary metabolite production in some important plant pharmaceuticals.

2. Generic Pathways for Plant Response to Abiotic Stresses

According to our current knowledge about stress signaling pathways, the generic signaling pathway for any given abiotic stress can be divided into the following major steps: signal perception, signal transduction, stress-responsive gene expression and the activation of physiological and metabolic responses [23]. When plants are exposed to
drought or salinity stresses, they produce signals that are perceived at the membrane level by ion channels, membrane receptors, receptor-like kinases and many other proteins. Upon perception, complex intracellular signals are then initiated and cause the generation of many messengers such as Ca$^{2+}$, reactive oxygen species (ROS), phytohormones and other stress signaling molecules [24–26]. Those signals are then transduced further inside the cell and interact with other molecules that will ultimately lead to the induction of the stress-induced gene that encodes for proteins that will particularly enhance the plant’s tolerance to abiotic stresses directly or indirectly. The plant response to abiotic stresses can be described as the coordination of expression of genes that encode for a variety of products that contribute to the plant’s adaptation to abiotic stresses [27,28]. Those products could carry out any function that supports the plant in the adaptation to the stress; they could be, for instance, secondary metabolites that carry out essential protective functions against abiotic stresses, transcription factors that facilitate the activation of second-level stress-inducible genes or any other functional or regulatory molecules [29].

Based on the protein products of the stress-inducible genes, they can be divided into two functional categories: (1) genes that encode for products that directly affect the plant’s tolerance to salinity and water deficit stresses, such as heat stress proteins (HSPs) or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free-radical scavengers [30], and (2) stress-responsive genes that encode for protein products that influence the abiotic stress resistance by regulating the expression of other downstream target genes and modulate the signal transduction cascades. The latter category includes genes that may produce phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene [7]. It also includes genes that encode for other signaling molecule products that contribute to the signaling cascade regulation of gene expression, such as the mitogen-activated protein kinases (MAPKs), calcium-dependent protein kinases (CDPKs) and a great number of stress-inducible transcription factors (Figure 1).

Figure 1. Generic pathway for plant response to salinity and drought stresses in Arabidopsis thaliana cell. Salinity and drought elicit Ca$^{2+}$ signals through the SOS pathway to achieve ionic homeostasis. When the ions are increased, it is first perceived by the ion stress sensors, and then the Ca$^{2+}$ levels are increased. The Ca$^{2+}$ binds to the SOS3 protein and then interacts with the SOS2 protein kinase. The Ca$^{2+}$–SOS3–SOS2 complex phosphorylates and activates the SOS1 membrane protein, which will cope with the ionic stress. This change in cytoplasmic Ca$^{2+}$ level is sensed by calcium sensors (CDPKs, CaMs, CMLs and CBLs) that interact with their downstream signaling components, which could be RBOHD or transcription factors. The activation of RBOHD leads to the generation of ROS molecules, which in turn aid in the signaling mechanism through the interaction with many molecules such as phytohormones or several kinases. Those phytohormones (ABA) or the protein kinases interact with the stress-inducible TFs. Those stress-inducible TFs carry specific DNA-binding domains that bind to specific regulatory sequences found upstream of the stress-inducible gene and initiate transcription. Those genes will lead to several physiological and chemical changes that would enhance the plant’s tolerance to salinity and drought stresses.
2.1. \( \text{Ca}^{2+} \) and ROS Regulation during Salinity and Drought Exposure

The mechanism of genetic regulation in plants upon the exposure to drought or salinity as an abiotic factor involves the activation of several intricate signaling transduction pathways. There are many intracellular signaling molecules that are involved in the plant’s adaptation mechanism to salinity and drought stresses in which they interact together in a complex manner in order to produce a final protective role. Secondary messengers such as ROS and \( \text{Ca}^{2+} \) are considered well-known and significant signaling molecules that are active at the first steps of the plant’s response to abiotic stresses [31].

2.2. \( \text{Ca}^{2+} \) Ions

As reported by many articles, the most important universal secondary messenger is calcium ions (\( \text{Ca}^{2+} \)). As mentioned before, salinity and drought stresses cause ionic and osmotic stresses for the exposed plant. Hence, the plants adapt to the stresses by retaining the cellular homeostasis by dealing with osmotic and ionic stresses. It was discovered that the Salt Overly Sensitive (SOS) genes are a primer mediator for the ionic hemostasis in plants. SOS3 gene encodes for a protein that acts as a receptor for cytosolic \( \text{Ca}^{2+} \) [32]. Increased levels of \( \text{Ca}^{2+} \) lead to the activation of the cascade of events that would activate the genes responsible for protecting the plant from salt stress. For instance, the \( \text{SOS1} \) gene encodes for the plasma membrane Na\(^+\)/H\(^+\) antiporter, and the \( \text{SOS1} \) gene is mediated by the \( \text{SOS2} \), which encodes for a calcium-dependent protein kinase (CDPK) [32–34]. The plasma membrane Na\(^+\)/H\(^+\) antiporter aids in protecting the plant from salinity stress by maintaining the osmotic balance by compartmentalizing Na\(^+\) and chloride into vacuoles or extruding the excessive toxic Na\(^+\) to the apoplast or surrounding tissues [35–38]. Along with SOS, \( \text{Ca}^{2+} \) ions cooperate with other signaling molecules, including calcium-dependent protein kinases (CDPKs), which are considered very important phosphorylating agents in the plant response to abiotic stresses [39].

CDPKs play important roles at the cellular and molecular level in plants: increasing the resistance and adaptation to abiotic stress and modulating metabolic pathways [40]. The CDPKs play a significant role in the biosynthesis of important organic molecules by phosphorylating and activating a variety of biosynthetic metabolic pathways. Allwood discovered that a key enzyme in the defense response against abiotic stress, known as the phenylalanine ammonia lyase (PAL) enzyme, could be phosphorylated by a CDPK isoform [41], indicating its connection to the secondary metabolite biosynthetic pathways and abiotic stress resistance.

2.3. Reactive Oxygen Species (ROS)

During abiotic stresses, such as salinity and drought, overproduction of ROS results in perturbation of the cellular redox state and thus poses the threat of developing the toxic event that is known as oxidative stress. However, ROS are usually present at a low level in many organelles, and they act as significant secondary messengers in the plant’s response to abiotic stresses and secondary metabolism. They are considered very effective secondary messengers due to their spatiotemporal flexibility [31].

There is a synergistic effect in the plant’s defense system between \( \text{Ca}^{2+} \) and ROS signaling [42,43]. The respiratory burst oxidase homolog (\( \text{RBOH} \)) gene family encodes for an enzyme known as the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This product enzyme is considered the major contributor of de novo synthesized ROS extracellularly. The \( \text{RBOH} \) gene is regulated and activated by the previously mentioned calcium-dependent protein kinase (CDPK), which facilitates its phosphorylation. Thus, in order to produce more ROS, \( \text{Ca}^{2+} \) levels ought to be increased. Vice versa, elevated levels of ROS lead to the increased concentration of \( \text{Ca}^{2+} \) ions by the interaction of the H\(_2\)O\(_2\) and hydroxyl radicals with the \( \text{Ca}^{2+} \)-permeable cation channels such as the voltage-independent \( \text{Ca}^{2+} \)-permeable cation channels (VICCs) and the hyperpolarization-activated \( \text{Ca}^{2+} \)-permeable cation channels (HACCs). This results in a noticeable transient increase in
the cytosolic Ca\textsuperscript{2+}, which is involved in several fundamental physiological and regulatory activities [26,44,45].

Similar to Ca\textsuperscript{2+}, ROS interact with other signaling molecules such as the MAPKs. The mitogen-activated protein kinase is one of many important signaling molecules that initiate cascades of events that are involved with transducing stress-related stimuli such as enhanced levels of ROS or calcium signaling. It was discovered that ROS upregulate the activity of the ANP1 kinase in Arabidopsis [46] and its functional homolog NPK1 in tobacco [43], which trigger the downstream MAPK cascades. Several molecular genetic experiments indicated that ANP1 is responsible for the regulation and activation of AtMPK3 and AtMPK6 [39]. The protein kinase products of MPK3/MPK6 are responsible for the direct phosphorylation of the transcription factors associated with the plant’s defense system, such as WRKY and ERF. Many other studies have proved the association between ROS and MAPKs in several plant models, indicating their function in the plant’s response to abiotic stresses [47,48].

3. Phytohormonal Regulation in Salinity and Water Deficit Stresses

Phytohormones, e.g., abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene [7], play critical regulatory tasks in the plant’s response to abiotic stresses and metabolic pathways. Each hormone plays a variety of functions, and all together act in a concerted way rather than a stand-alone action. Generally, hormones are synthesized at low concentrations in the body of the plant, and they function as a controlling agent in different aspects of developmental and growth events throughout the plant’s cycle of life [43].

3.1. Abscisic Acid

ABA contributes to a variety of regulatory networks involving plant’s response to drought and salinity stresses that cause osmotic and ionic stresses. Under conditions of osmotic stress, abscisic acid is generally considered as a stress signaling hormone, and the expression of stress-responsive genes in plants is primarily regulated by ABA-dependent and ABA-independent pathways based on the cis-acting regulatory elements present in the promoter regions of the stress-responsive genes [49,50]. The cis-acting elements are regulatory sequences found at the promoter region of many genes, and they are important because they act as physical targets for many transcription factors in order to activate the downstream target genes. The transcription factors usually carry a specific DNA-binding domain that binds to particular cis-regulatory elements [51]. In the ABA-dependent gene expression, the cis-acting element is the ABA-responsive element (ABRE) and involves the function of the ABA-dependent ABRE-binding protein/ABRE-binding factor (AREB/ABF) transcription factors that target the sequence known as G-box-like cis-acting element with the sequence of ACGTGG/TC [52].

Abscisic acid phytohormone regulates the activation of calcium channels that in turn lead to the activation of the previously mentioned enzyme NADPH oxidase, which has the ability to produce de novo ROS molecules. Moreover, ROS interacts with ABA and other hormones for the main purpose of defending the plant by regulating the signaling cascades and the expression of many stress-responsive genes. Such genes’ products are antioxidant enzymes with ROS termination functions such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) [43].

3.2. Ethylene

Ethylene is a gaseous molecule and a well-studied plant phytohormone that carries out important molecular modulation responsible for enhancing the tolerance of the plant to abiotic stress and regulating several metabolic pathways for secondary metabolite biosynthesis. Under salinity and drought stresses, the accumulation of ethylene was observed in many plants. A considerable body of evidence suggests that ethylene functions by monitoring gene expression as a part of its signal transduction pathway. In the absence
of ethylene, very important genes that contribute to the plant’s response to stress will not be expressed; on the contrary, they will be degraded by E3 ubiquitin ligase [53,54]. Those genes are known as ethylene insensitive 2 (EIN2) and ethylene insensitive 3 (EIN3); they are two key intracellular ethylene-mediated gene regulators in the plant’s defense response. With ethylene, EIN2 accumulates and thus facilitates the accumulation of the EIN3 proteins. The EIN3 proteins have a very important role in the genetic activation of a crucial stress-inducible transcription factor known as ethylene response factor 1 (ERF1) [53,55]. The ERF1 then assists as a transcription factor and recognizes a GCC cis-acting element in the promoter regions of ethylene-responsive genes and triggers transcription. This finding shows only one example of how ethylene as a plant hormone contributes to regulating gene expression in the plant’s response to abiotic stresses such as drought and salinity.

3.3. Jasmonic Acid and MeJA

Plant produce jasmonic acid (JA) as a plant hormone to regulate a variety of plant processes, including response to abiotic stresses and regulation of the biosynthetic pathways of many secondary metabolites [12]. JA or JA-Ile signaling results in the activation of many TFs that regulate the expression of a great number of target genes through specific binding to particular cis-acting elements. Moreover, jasmonic acid and the methyl ester form of jasmonic acid (MeJA) have been proved to be able to act as an elicitor for the production of several secondary metabolites such as alkaloids, flavonoids, other phenolic compounds and many other compounds [56].

4. Transcriptional Regulation during Salinity and Drought Stresses

Transcription factors are regulatory proteins that recognize particular regulatory sequences of bases located upstream of the coding region of the gene and thus control the transcription process. Every transcription factor is identified by conserved and specific DNA-binding domains that are specific for several cis-acting elements. By binding to cis-regulatory sequences, these proteins facilitate either the activation or repression of the transcription of downstream genes. With regards to transcriptional regulation of the transcription factors in response to abiotic stress, studies have found that many regulatory transcription factors play essential roles in multiple abiotic stress responses by regulating a large spectrum of downstream genes; hence, these genes are known as stress-inducible or -responsive genes [57].

In the last few decades, considerable research has been conducted to identify and characterize various TFs involved in plant abiotic stress responses and metabolic pathways either in abscisic acid (ABA)-dependent pathways or ABA-independent pathways, such as AP2/EREBP, MYB, bHLH, WRKY, NAC and bZIP [52,58]. Arabidopsis thaliana utilizes over 5% of its genome to code for over 1500 TFs, roughly 45% of which are from families that are only specific to plants [59]. In this review, detailed information regarding the transcriptional regulation of a variety of transcription factors, including the superfamilies AP2/EREBP, MYB and bHLH, is provided.

4.1. AP2/EREBP

The APETALA2/ethylene-responsive element binding protein (AP2/EREBP) family is a family of transcription factors thought to have a significant role in regulating gene expression at the transcription level [23,60,61]. These TFs have a highly conserved DNA-binding domain known as AP2/ERF DNA-binding domain. This AP2/ERF (APETALA2/ethylene-responsive element binding factor) is a domain consisting of 50–70 amino acids and is found in many proteins in the plant kingdom, such as A. thaliana ERF1, tobacco EREBP's (homolog of the ERF), A. thaliana AP2, A. thaliana C-repeat/dehydration-responsive element (DRE) binding factor 1 (CBF1 or DREB1) and DREB2 and Arabidopsis thaliana and maize abscisic acid (ABA)-insensitive 4 (ABI4) proteins [54,61].

Upon stresses, the AP2/EREBP superfamily are induced through cis-acting elements present at their promoter region. These regulatory elements include GCC box, ABRE, EBS,
HSE, LRT and many other unknown binding sites that respond to a variety of abiotic stress stimuli such as AREBs, EIN3 and other transcription factors [61,62]. Thus, the transcriptional regulation pathways of this superfamily of transcription factor proteins are both ABA-dependent and -independent. For instance, promoter analysis studies on the DREB subfamily determined that it contains the most diverse cis-acting elements that are related to abiotic stresses, including ABRE, MeJA response, TCA, ERE, MBS, HSE, TC-rich and LTR motifs. This finding suggests that those transcription factor-encoding genes are the most frequently expressed and involved in the signal transduction pathways related to abiotic stresses [51,54]. It also suggests that this subfamily is regulated through ABA-dependent pathways due to the presence of the ABRE cis-acting element and through the ABA-independent pathway due to the availability of the other cis-regulatory elements.

The DREB 2 subfamily of the AP2/EREBP superfamily can regulate the expression of multiple responsive to dehydration (RD) genes by interacting with a GCC-like box called the DRE/CRT cis-acting element found at the promoter region of many stress-inducible genes in a variety of plant species (e.g., RD29A, RD17, ERD10) [63–65]. On the other hand, the ethylene-responsive factor (ERF) subfamily, which also belongs to the AP2/EREBP superfamily, activates an array of stress-responsive genes through the interaction with cis-regulatory sequences that are found upstream of the coding region of many stress-inducible genes such as the ethylene-response element (ERE) or GCC box with the core sequence of AGCCGCC, jasmonic acid- and elicitor-responsive element (JERE), coupling element 1 (CE1) and CT-rich element [6,55,66]. For instance, it was found that a certain ERF encoded by the *ERF74* binds to the RbohD promoter region and triggers its expression [54,67] and hence participates in the defense mechanism against salinity and drought by generating ROS that act as signaling molecules. There are many other pieces of evidence suggesting the solid regulatory effect of the AP2/ERF family of TFs in the plant response to abiotic stresses [68].

4.2. MYB

The MYB family of transcription factors is one of the largest TF families in plants. A growing body of evidence has demonstrated the regulatory effects of the MYB family on many biological and biochemical activities such as defense and stress response regulation (Wu et al., 2019), primary and secondary metabolism regulation (Dubos et al., 2010; Wu et al., 2019) and the regulation of other plant-related developmental mechanisms. The MYB family of TFs is characterized by the presence of a conserved MYB DNA-binding domain.

A transcriptional regulatory network analysis of MYB TF family genes in rice was conducted by Smita et al. [69] in order to identify new links and characterize the interaction between the MYB family and the downstream target genes. In their study, they observed around 40 putative target genes for OsMYB that contain at least one MYB-binding region in their promoter. Out of the 40 target genes, 27 were found to have a high number of MYB-binding regions involved in drought-inducibility (MBS, CAACGG and TAACTG). This finding implies that the OsMYB genes are strongly involved and have great regulatory roles in drought response at the transcriptional level [69]. Studies have shown that *AtMYB2*, *AtMYB74* and *AtMYB102* MYB genes are upregulated by drought stress [70], which indicates that they play a role in the stress response regulation to protect the plant against the harmful effects of water deficit. Additionally, a study by Hasan et al. [71] investigated the possibility of *MYB44* interacting directly with ABA signaling in cotton species. They concluded that the *MYB44* gene encodes for a TF that interacts with ABA receptor proteins such as PYLs, which bind to PP2Cs. Hence, the MYB44 has been determined to be a regulator in the ABA-dependent signaling pathway that is related to the activation and repression of the SNF1-related protein kinases 2 (SnRK2s) in which it phosphorylates several stress-inducible genes. *AtMYB20* enhances salt tolerance by repressing the expression of PP2Cs [71,72].
4.3. Basic Helix–Loop–Helix (bHLH)

The bHLH family of transcription factors is the second largest family in plants after the MYB family. In Arabidopsis, 162 members of bHLH have been identified, and 167 have been identified in rice plants [73]. As the name suggests, the bHLH family of transcription factors contains a highly conserved basic/helix–loop–helix unique structural domain consisting of two parts: basic amino acid and the helix–loop–helix region (HLH). Findings have determined that the bHLH protein predominantly attaches to the core DNA sequence motif called E-box (5-CANNTG 3), where N could be any nucleotide; the most common form of E-box is the G-box (CACGTG), which is also a binding sequence for the MYC family [74,75].

A growing body of evidence proves that the bHLH TFs are strictly involved in plants’ response to a variety of abiotic stresses such as drought and salinity [74,76]. Cui et al. [77] conducted a transcriptome-wide expression analysis of bHLH genes from tea (C. sinensis) and revealed that a total of 39 CsbHLH were upregulated under the exposure of the plant to drought stress. Moreover, transcriptomic studies in Arabidopsis roots under salt exposure have shown that at least 15 bHLH genes are upregulated in response to the stress, which implicates their functional role in salt stress responses in plants. There is other evidence suggesting the crucial involvement of the bHLH transcription factor family in plants’ response to abiotic stresses.

5. Stress-Inducible Genes

The stress-inducible genes, which are usually activated by any of the stress-inducible transcription factors, play a direct protective role that causes the plant to defend itself against the harmful and damaging effects of the drought and salinity stresses. These target genes increase the tolerance of the plant against salinity and drought by regulating many biological and biochemical activities [35]. For instance, these genes can produce osmoprotectants, proline [78], osmolytes, detoxifying agents, antioxidant enzymes [4] and many other agents that contribute to the adaptation of the plant to drought and salinity stresses.

Osmolytes are some of the most important molecules involved in abiotic stress tolerance. They are very important endogenous metabolites that are known to carry out many functions in order to maintain the osmotic pressure in the plant [79]. Proline is the most widely known type of osmolyte, and the biosynthesis of the proteinogenic amino acid proline occurs in the cytosol and chloroplast. In Arabidopsis, it is controlled by the P5CS2 and P5CS1 genes based on the location of synthesis: cytosol or chloroplast, respectively [80]. Under salt stress, calmodulin (CAM) has an important role in the activation of an MYB family transcription factor (MYB2). The MYB2 binds to a cis-acting element in the promoter of the P5CS1 gene; thus, it plays a significant role in the biosynthesis activity of proline. Moreover, promoter analysis studies of AtP5CS1, AtP5CS2 and AtP5CR indicated that their promoter regions contain ABA-responsive elements as cis-regulatory elements, which indicate their connection to the ABA-dependent pathway [24,69,81].

Environmental stress increases the level of reactive oxygen species (ROS), which are useful in signaling at low concentrations but toxic at a higher level. To scavenge and detoxify the ROS molecules, the plant recruits several enzymatic and nonenzymatic antioxidant systems in order to eliminate the harmful effect of the ROS. The major ROS-scavenging enzymes of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) [82] and peroxiredoxin (PrxR) [47]. The nonenzymatic antioxidants such as ascorbic acid, tocopherols, flavonoids and carotenoids provide the cells with high-performance machinery for fighting oxidative stress [12,83]. Those antioxidant genes are regulated during abiotic stress via signaling and transcription factor regulation. For instance, transgenic cotton overexpressing the potato DREB2 TF showed enhanced expression of antioxidant genes (SOD, CAT, etc.) under exposure to water deficit [84]. Studies have shown that there is a relationship between the plant hormones ethylene and JA and the upregulation of several antioxidant-related genes, whether their products are
enzymatic or nonenzymatic. The whole transcriptional regulation of the antioxidant genes is not fully clear yet, and studies are still being conducted for the sake of having a complete understanding of the whole regulatory mechanism of the antioxidant activity [83].

6. Secondary Metabolite Production and Elicitation Technique

Plants produce thousands of organic compounds that are divided into two large groups: primary and secondary metabolites. Primary metabolites are essential for plant growth and development, and the majority appear to be common in all plants. The plant secondary metabolites are often referred to as compounds that have no essential role in the plant’s development and growth yet possess other important roles such as plant defense and adaptation against abiotic stresses. Plant secondary metabolites are considered very valuable and unique sources for pharmaceuticals, food additives, flavors and industrially important biochemistry [85].

Many studies agree that the accumulation of particular secondary metabolites is achieved by exposure to abiotic stresses. Secondary metabolites are connected with other chemicals that are involved in salinity and drought stress resistance, including calcium, ABA, SA, JA, ethylene and other signaling molecules [86,87]. Some of the predominant secondary metabolites in *Camellia sinensis* are the catechins, which are a type of polyphenolic flavonoids [88]. Studies on catechins have found that these metabolites are able to either generate or scavenge reactive oxygen species [89]. In the matter of abiotic stress, as reported above, ethylene is a critical plant hormone in signal transduction and resisting the adverse effect of the stress. A study conducted on *Camellia sinensis* showed that the treatment of a precursor of ethylene known as 1-aminocyclopropane-1-carboxylic acid (ACC) showed enhanced accumulation of phenolic compounds. Additionally, the antioxidant enzyme activity of SOD, CAT and POX enzymes was decreased, whereas that of the APX enzyme was increased due to the precursor treatment [88]. These findings indicate a presumable implication of induction of the secondary metabolites in regulating the balance of ROS in the plant via ethylene signaling. This information indicates that the secondary metabolites contribute significantly to the plant’s resistance to abiotic stresses.

Along with their contribution to preventing harmful effects of salinity and drought stresses, they also possess economically important values such as acting as a source of food or traditional medicine. Many medicinal plants are sources of very important drugs such as alkaloids from Catharanthus (for chemotherapy) [90], melatonin from *Tanacetum parthenium* [91] and antioxidant secondary metabolites from many plants. Thus, understanding how the economically significant secondary metabolites are produced is very important.

Although plant phytochemicals have powerful beneficial values as plant protectants and human therapeutic values, their production in plants is often at low concentrations in different organs of the plant [92]. Therefore, scientists focus on the development of a variety of enhancement tools for the production of secondary metabolites using cell and molecular genetic approaches. One famous strategy used to enhance the production of phytochemicals in plants is the elicitation technique. An elicitor is globally used for “any physical or chemical agent capable of inducing or stimulating defense response in plant cells/tissues via production of secondary metabolites” [93,94]. These elicitors modulate the regulation of the biosynthetic pathways that are involved in the natural biosynthesis of significant phytochemicals in the plants. An elicitor could result in the induction of transduction pathways that lead to the production of the secondary metabolite.

There are many types of factors that could be considered as elicitors for the plant enhancement to produce secondary metabolites. An elicitor could be endogenous or exogenous. An endogenous elicitor is one produced by the plant without foreign participation. An exogenous elicitor originates from outside the plant. In this review, we focused on the elicitation of abiotic factors such as salinity and water deficit stresses [94]. Those abiotic stresses could act as physical or chemical elicitors. For instance, a chemical elicitation caused by high salinity is due to the increased levels of NaCl absorbed by the plant, which would lead to osmotic and ionic stresses. A physical abiotic elicitor such as temperature is
usually accompanied by drought stress. NaCl, or salt, has been widely used as a type of chemical elicitor that has presented remarkable results considering increased production of secondary metabolites, as mentioned before. Additionally, plant stress-regulatory phytohormones such as MeJA, jasmonic acid and brassinosteroids are considered well-known endogenous elicitors for enhanced phytochemical production (Figures 2 and 3). However, studies have shown that the exogenous application of JA has a profound effect on the modulation of SM biosynthetic pathways, hence enhancing the production of secondary metabolites, as is exemplified in the following section.

Studies have shown that secondary metabolite production is somehow dependent on abiotic factors. Scientists have observed that under exposure to abiotic stresses, plants tend to produce many secondary metabolites that contribute powerfully to defending the plant from the damaging effects that are caused by the abiotic stress. Therefore, in many references, scientists concluded that abiotic stresses are considered elicitors that cause the production of secondary metabolites by modulation the biosynthetic pathways at the biochemical or transcription level. Thus, an abiotic elicitor, whether it is a specific stress-inducible gene overexpression, stress-inducible transcription factor, growth hormone or any molecule that is activated during stress and causes secondary metabolite production, can be used to modulate and regulate the biosynthetic pathways of pharmacologically active phytochemicals in medicinal plants.

![Diagram of the vinblastine biosynthetic pathway](https://example.com/diagram.png)

**Figure 2.** (A) The vinblastine biosynthetic pathway. (B) Jasmonic acid elicitation of vinblastine in *Catharanthus roseus*. JA content is increased when the plant is exposed to salinity or drought stress or both together. Then, the AP2/ERF TF known as ORCA3 is activated and binds to the regulatory *cis*-element (JERE) that is found upstream of the STR gene that encodes for a key regulatory enzyme in the vinblastine biosynthesis pathway.
level leads to the activation of two AP2/ERF transcription factors (AaERF1 and AaERF2) that bind to the CBF2 and RAA cis-acting elements that are found on the promoter regions of the ADS and CYP genes that are major components of the artemisinin biosynthesis pathway.

Figure 3. (A) The artemisinin biosynthetic pathway. (B) Jasmonic acid elicitation of artemisinin in the plant Artemisia annua. JA content is increased when the plant Artemisia annua is exposed to salinity or drought stresses. The increased JA level leads to the activation of two AP2/ERF transcription factors (AaERF1 and AaERF2) that bind to the CBF2 and RAA cis-acting elements that are found on the promoter regions of the ADS and CYP genes that are major components of the artemisinin biosynthesis pathway.

7. Biosynthetic Pathways of Transcription Factors, Signaling Molecules and Secondary Metabolites

The significance of metabolic engineering resides in the fact that it will unleash the capabilities of improving the production of beneficial secondary metabolites or focusing on the improvement of the plant’s resistance to abiotic stresses such as salt and drought stresses. There is a strong correlation between stress-induced genes and secondary metabolite production. However, some secondary metabolite-encoding genes are considered stress-induced genes since they carry out protective roles and are induced during exposure to stress.

Primary signals in most environmental stresses involve reactive oxygen species (ROS); hormones such as ethylene, JA and abscisic acid (ABA); transcription factors (TFs); and other signaling molecules. TFs, which are DNA-binding proteins, have an indispensable job in the regulation of secondary metabolite production in plants because they control the expression of many genes that play multiple roles in the metabolic pathway. However, it was demonstrated by studies on many plants that JA contributes to the transcriptional activation of many TFs that are used to boost the expression of specific biosynthetic genes for secondary metabolites. For instance, MYC2, a bHLH-type transcription factor, has been identified as the regulatory center of the JA-signaling pathway, thereby affecting secondary metabolite accumulation in various plant species including Artemisia annua, Nicotiana tabacum and Catharanthus roseus [6,95]. Additionally, the AtMYB12 gene, which encodes for a stress-inducible transcription factor, is responsible for the modulation of the expression of genes involved in the phenylpropanoid pathway which would result ultimately in enhanced production of secondary metabolites. A study conducted by Shkryl [96] suggested that overexpression of the A. thaliana gene that encodes for a calcium-dependent protein kinase (AtCPK1) in the medicinal plant R. cordifolia caused a substantial increase in the anthraquinone content production in the transgenic plant. The calcium-dependent protein kinases are strongly induced when plants are adapting to drought and salinity stresses, as reported above.

Exogenous application of jasmonates, specifically MeJA, should enhance production of the secondary metabolite flavonoid (anthocyanin) in the model plant Arabidopsis thaliana
and other plants. MeJA participates in the regulation of the monoterpene indole alkaloid (MIA) biosynthetic pathway in the well-studied medicinal plant *C. roseus* by coordinately inducing the expression of biosynthetic-related genes (Figure 2). Promoter analysis study showed that specific AP2/EREBP transcription factors known as octadecanoid-derivative responsive Catharanthus AP2-domain protein 1 (ORCA1) and ORCA2 have a strong relation to the expression of important MIA biosynthetic genes [40,97]. ORCA2 showed strong relation to the MeJA-responsive MIA biosynthesis genes, such as the strictosidine synthase (STR) gene (Figure 2). Further research has identified CrMYC2, another transcription factor belonging to the bHLH family of transcription factors, as a major activator of MeJA-responsive ORCA3 gene expression. An experiment conducted by Zhang [95] involving knockdown mutant of the CrMYC2 expression level by RNAi technique showed results indicating that expression levels of ORCA3 and ORCA2 were reduced significantly. This experiment suggests that the gene regulation of the alkaloid biosynthesis in *C. roseus* is modulated by a cascade of transcription factors belonging to different families (AP2/EREBP and bHLH) [95,98].

Another example of a JA-responsive gene that participates in secondary metabolite production is found in *Artemisia annua* (Figure 3) [97,99]. *A. annua* produces artemisinin, a natural product drug for malaria treatment. It was found that two genes encoding AaERF1 and AaERF2, which also belong to the AP2/EREBP family of transcription factors, are responsive to JA. Subsequently, those transcription factors were then found to bind to the promoters of genes that encode for key regulator enzymes in the artemisinin biosynthesis pathway, which are known as sesquiterpene synthase amorpha-4,11-diene synthase (ADS) and P450 monoxygenase CYP71AV [97]. In support of this finding, researchers also observed that overexpression of AaERF1 and AaERF2 in transgenic *A. annua* plants increased the expression of the biosynthetic enzymes (Figure 3) [97]. However, it is obvious that these facts indicate that the jasmonates as plant phytohormones interact with transcription factors and hence confer great control over the expression of many target genes, especially in secondary metabolite production.

The bHLH and MYB TF family plays a significant role in the regulation of many biosynthetic pathways that are involved in secondary metabolite metabolism and production [6,30,72]. MYB proteins play roles in controlling phenylpropanoid metabolism; for example, in *Petunia* flowers, where the AN2 gene product is required for anthocyanin production, it has been shown to encode an MYB-related product [6,72,100,101]. AtMYB75, AtMYB90, AtMYB113, AtMYB114, production of anthocyanin pigment 1-4 (PAP1-4) and TT2 (AtMYB123) are known to regulate anthocyanin and proanthocyanidin biosynthetic pathways [27,72,100,102]. The anthocyanins, proanthocyanins and flavonols belong to one of the largest groups of secondary metabolites that are distributed widely in plants; flavonoids. Flavonoids as secondary metabolites confer many protective functions against abiotic stresses such as signaling and antioxidant activity [12]. In a study conducted by Wang [103], the *Vv bHLH1* gene from grapes was overexpressed in other plants such as Arabidopsis for the sake of increasing the content of valuable flavonoids and thus improving the tolerance of the transgenic plants under abiotic stresses such as drought and salinity. It was also found to increase the content of ABA, which is important for the regulation of many important signaling pathways that are involved in the abiotic stress defense mechanism.

8. Comprehensive Gene Expression and Transcriptome Analysis Solution in Medicinal Plants

There are many gene mining approaches employed for the elucidation of the biosynthetic pathway regulation that occurs in plants; they involve several -omics studies such as transcriptomics, proteomics, metabolomics and bioinformatics approaches. The transcriptome is the complete set of all RNA available in a particular cell, including noncoding RNAs [104]. Transcriptome analysis is a molecular genetic strategy that enables scientists to describe and quantify the transcriptome of a particular organism with the intention of deciphering the complex genetic regulation at the transcriptional level. The develop-
ment of biotechnology has enabled the utilization of a variety of high-throughput DNA sequencing and identification methods for mining and quantifying transcriptomes, including PCR amplification, microarray analysis and next-generation sequence (NGS)-based RNA-seq [105].

In the field of functional genomics, the transcriptome analysis approach has played a crucial role in decoding the complexity of gene regulation in plants. Numerous transcriptomes have been sequenced in plants using a variety of technological approaches that allowed considerable identification and quantification of the transcriptome, including hybridization- or sequencing-based methods [106–108]. Hybridization-based technologies, such as DNA microarrays, are used in a powerful technique that requires a preselected RNAs library for the fulfillment of a simultaneous hybridization with a fluorescence agent. The intensity levels of the hybridization signify and indicate the expression levels of the genes [109,110]. On the other hand, sequencing-based methods, such as expressed sequence tag (EST), serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS), use sequencing strategies that do not require the involvement of a preselected RNA library for obtaining results. The MPSS is also known as deep sequencing or next-generation sequencing (NGS) [111]. NGS has become the revolutionary approach in biology due to its remarkable ability to collect and obtain valuable data in a short time, and it has been considered a very useful technique for studying the transcriptome of plants and other organisms. NGS could be coupled with other techniques such as the RNA-seq approach. RNA-seq is a high-throughput sequencing technique that provides a comprehensive gene expression profile of each sample with the potential to quantify and annotate all genes and isoforms [112]. RNA-seq is a sequencing-based approach that is coupled with next-generation sequencing tools for the fulfillment of a variety of objectives. Using RNA-seq technology enabled scientists to perform several experiments such as measuring gene expression levels, obtaining differential expression profiles (DEG), identifying gene structural properties (transcription start site, exon–exon junctions, etc.), identifying novel transcripts, identifying transcript isoforms and determining allele-specific expression [113].

A transcriptome analysis study conducted by Verma et al. [90] provided large-scale transcriptome profile data for understanding the specialized metabolism of C. roseus that includes the metabolic pathways for the pharmaceutically useful secondary metabolites. The medicinal plant Catharanthus roseus accumulates a wide range of terpenoid indole alkaloids, which are well documented for their medicinal purposes and include the well-known drugs vinblastine and vincristine, which are widely used in anticancer chemotherapies. The plant is known to treat diabetes also, due to the hypoglycemic properties in its tissue extracts [114]. In general, the natural product of this plant is known to be of significant value medicinally. Using the RNA-seq technique, scientists were able to present a great body of valuable knowledge regarding the gene regulation of the plant [115].

Gene expression analysis using RNA-seq data revealed that many of the genes involved in the TIA pathway are differentially expressed in root and leaf tissues, which implies that these beneficial alkaloids are synthesized primarily in the roots and the leaves [116]. Quantitative RT-PCR analysis was also performed for 10 genes that are involved in the TIA pathway (Figure 1), showing results that were strongly correlated with the RNA-seq data and validated their differential expression in the leaves and roots. The genes that are upregulated in the leaves are related to the photosynthesis process, whereas genes that encode for the TFs were found in the roots. Using a heat map of 1861 upregulated genes, it was discovered that 148 genes encode for TFs, most of them belonging to the AP2-EREBP, HB, MYB, NAC and WRKY families. Moreover, TIA pathway genes were upregulated in hairy root cultures for which the methyl ester form of jasmonic acid (MeJA) acts as an elicitor.

9. Conclusions

In conclusion, exposure of the plant to salinity and drought, abiotic stresses, leads to a severe decrease in the potential of the plant for growth and productivity. In order to fight
the harmful effect of stress, the plant manages a number of regulatory events involving secondary messengers, signaling proteins, hormones and a variety of stress-inducible transcription factors and their putative target downstream genes. The communication and regulation of the machinery that controls the resistance of the plant to salinity and drought stress is a very complicated subject to cover and is not fully understood. Many studies have shown that the overexpression of regulatory genes, such as TFs, proline synthesis genes, antioxidant genes and many other stress-inducible genes, plays a significant role in enhancing plant tolerance to salinity and drought stresses. However, to control the expression of multiple genes, studies have shown that genetic engineering and overexpression of TFs provided the necessary stimulus and increased tolerance; thus, TFs became potential candidates to target in efforts to enhance the resistance of the plant to salinity and drought stresses. Moreover, genetic engineering and molecular techniques should be employed in attempts to understand the relationship between the phytohormones and major steps in a specific biosynthesis pathway; this will provide more detailed information about the genetic regulation of the pathways. The RNA-seq approach has become a widely used and indispensable method for studying gene expression and obtaining a transcriptome profile of model and non-model plant species. As sequencing technologies are continuously being improved, new RNA-seq methods with better results are expected to emerge in the future. The recent development in sequencing technologies has already enabled the achievement and creation of complete genome and transcriptome sequences for a great number of plant species. The progress in sequencing technology and the application of RNA sequencing will enable scientists to conduct more projects that are related to obtaining comprehensive transcriptome profiles of many medicinal plants and identifying the up- and downregulated genes that are involved in the biosynthesis pathways of essential and economically valuable secondary metabolites. Acquiring more information about the molecular mechanisms of plants will eventually aid in finding solutions with economic and biological benefits that will provide prosperity for the world.

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