NPC Natural Product Communications

2008
Vol. 3
No. 8
1223 - 1238

Integration of Plasma Membrane and Nuclear Signaling in Elicitor Regulation of Plant Secondary Metabolism

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Received: May 2nd, 2008; Accepted: June 9th, 2008

The plant kingdom represents a valuable source of natural products of commercial interest. These compounds, named secondary metabolites, are not essential for the survival of plants, but confer them some advantages that allow adaptation to changes in their environment. Nevertheless, yields of secondary metabolites are low for commercial purposes, so it has become important to design strategies for increasing their production. Plants manage to adapt to physical changes in their environment, defending themselves against pathogen attack or herbivore wounding. Such aggressive stimuli, also known as elicitors, initiate signaling metabolic cascades that induce accumulation of certain secondary metabolites. Progress has been recently achieved in the understanding of signaling events originating from elicitation and related transcriptional regulation. These advances will allow maneuvering expression of key enzymes implicated in biosynthetic pathways of secondary metabolites, thereby enhancing their accumulation.

Keywords: plants, secondary metabolites, synthetic pathways, elicitors, membrane signaling, transcriptional regulation.

1. Basic Concepts of Secondary Metabolism

Metabolism is an intricate and complex network of chemical pathways involved in synthesis, conversion and breakdown of metabolites which represent the basis for life maintenance, and consequently they are called primary metabolites. Plants also exhibit a wide molecular diversity with many other compounds having no recognized role in fundamental life processes in the species that synthesize them and these compounds receive the name of secondary metabolites [1]. Nevertheless, they have important functions for plant survival. A few examples of these roles are the interaction of plants with their environment, plant defense systems and reproduction (attracting pollinators and in male fertility).

Besides the benefits that secondary metabolites confer to plants, many of them have proven to be useful to humans. Thus, several of these compounds are employed for the production of medicines, dyes, insecticides, to improve organoleptic quality of food (taste, color and smell), and as flavors and fragrances. The main advantages of using plants as sources of valuable compounds rely on the fact that costs of natural products are lower than those obtained by chemical synthesis and are made with negative environmental impact.

2. Elicitors

Despite the advantages that plants present over chemical synthesis, production levels of biological systems are limited for commercial purposes. In this context, intentional manipulation of molecular diversity [2] and the design of strategies which increase yields of natural products turned out to be an important issue. One emerging possibility is metabolic engineering, but this requires an understanding of the regulation of secondary metabolism at all levels (genes, enzymes, products, transport and compartmentalization), which is unfortunately still far from complete [3]. Another promising strategy for increasing production of valuable secondary metabolites is elicitation [4]. It is widely known that higher plants show various physiological and morphological responses to microbial attack and environmental changes. Such responses include the production of antimicrobial molecules, for example phytoalexins, proteinase inhibitors and reinforcement of the cell wall [5]. The compounds capable of triggering these responses are

Table 1: Classification of different classes of elicitors employed for enhancing secondary metabolism.

Elicitor type		
Biotic	Produced by pathogens	Chitosan, glucan polymers, oligosaccharides, glycoproteins (defined composition) Derived from fungal or yeast cell materials (complex composition)
	Derived from the plant	Cell wall fragments (pectin) Endogenous compounds release by the plant in response to the aggressive stimuli
Abiotic	Inorganic chemicals	Heavy metals
	Physical factors	Drought, high salt (osmotic stress), radiation, cold or heat shock

known as elicitors. In Table 1 a classification of different types of elicitors is presented. In the beginning, the term elicitor referred to those molecules capable of inducing synthesis of phytoalexins, but now it is used for molecules responsible for the initiation of any plant response. In other words, elicitation can be defined as a process that uses plant defense mechanisms to induce or enhance synthesis of valuable secondary metabolites. Indeed, many strategies have been assayed where either plant cell suspension cultures or mature plants are treated with different elicitors in order to improve yields of plant secondary metabolites of commercial interest.

As shown in Table 1, elicitors do not exhibit a chemical structure in common, moreover, they belong to a wide range of different classes of compounds and not all plants respond positively to the same elicitors [5].

In this review we discuss the initial events implicated in recognition of the external elicitor signal, intracellular signaling cascades that activate transcription factors, and the ensuing modifications in the transcriptional rate of key enzymes involved in the synthesis of secondary metabolites.

3. Plant Elicitor Receptors

It is widely assumed that an elicitor-initiated signal cascade requires a receptor entity capable of recognizing the elicitor in order to produce changes in secondary metabolism. Several pieces of evidence support the presence of receptor proteins located on the plant cell surface. The existence of proteins with receptor properties was reported in the early 80's in soybean membranes [6,7]. As mentioned before, not all plants are responsive to all elicitors. Plants also exhibit specificity regarding the signal transduction pathway activated by a certain elicitor. Altogether, this evidence supports the idea of a molecular entity placed on the cell surface capable of recognizing determined compounds and initiating a specific cellular response that allows plant survival, pathogen defense, synthesis of a secondary metabolite or adaptation to its environment. Molecular approaches allowed the identification of several putative receptors in plants. According to their structural characteristics they have been classified as receptor-like protein kinases (RLKs), histidene kinase receptors and receptors with different numbers of transmembrane domains [6-8]. Of interest, a large number of RLKs have been identified in the *Arabidopsis thaliana* genome, raising the possibility that these receptors may be implicated in perception of a broad range of stimuli and elicitors [9-11].

A well characterized receptor is the one for *N*-acetylchitooligosacharides (oligochitin, chitin oligosaccharides), compounds derived from the cell wall of filamentous fungi. It has been shown that oligochitin elicitors induce early cellular responses in rice cells, such as ion flux [12], biosynthesis of jasmonic acid [13], reactive oxygen generation [14], and the expression of several defense-related genes [15,16]. Shibuya *et al.* in a binding assay using an ^{125}I-labeled derivative of *N*-acetylchitooctaose as a ligand identified a high affinity binding site for an oligosaccharide elicitor in a plasma membrane preparation from rice cell suspensions [17,18]. Binding of the radio-labeled ligand was saturatable and reversible and the Scatchard plot analysis indicated the presence of a single binding site. Nevertheless, only a few cognate receptors for elicitors have been identified while other signaling elicitor-induced mechanisms remain unclear, as for example, oligo-α-galacturonides (OGAs) produced by degradation of homogalacturonan, a component of the plant cell wall. To date, no confirmed plant receptor for OGAs has been cloned [6]. It has been proposed that OGAs do not act through a receptor; instead their activity seems to be due to their physical properties. Apparently, their several negative charges produce physical changes on the plasma membrane [19], which in turn cause different responses. On the contrary, a RLK was shown to be induced in response to OGAs in soybean cells [20], but its exact role in the signal transduction pathways has not yet been established.

4. Early Metabolic Steps after Elicitor Perception

After detection of the elicitor in the cell surface, plasma membrane-bound enzymes (e.g. adenylyl cyclase, PLC) coupled to GTP binding (G) proteins

generate second messengers which initiate the cellular response. As described below, there is evidence that G proteins and the cAMP, IP_3, DAG and/or Ca^{2+} messenger systems are part of the early molecular events triggered during elicitation of plants. Mitogen-activated protein kinases (MAPKs) may also participate in elicitor signal transduction from the plasma membrane to the nucleus.

4.1 GTP binding proteins: G proteins play an important role in transducing elicitor signals across plant cell plasma membranes. The presence of GTP-binding proteins in plants includes small G proteins, heterotrimeric G proteins, and unconventional types of GTP binding proteins [21-23].

Regarding the structure of heterotrimeric G proteins, only one canonical Gα (GPA1) and Gβ (AGB1) subunits and possibly two Gγ subunits are encoded in the *Arabidopsis* genome [22]. In contrast, various gene copies for small G- proteins were identified [24]. These small GTP-binding proteins, ranging between 20 and 30 kDa and known as the Ras superfamily, share high homology in amino acid sequences [25].

G proteins have been involved in various cellular processes related to elicitation, as well as hormone signalling, development and defence responses [23,24]. There is evidence that heterotrimeric G proteins mediate elicitation. Anthraquinone production in *Rubia tinctorum* upon elicitation with chitosan was shown to be dependent on a G protein related to the Gαq family [26]. Also, elicitation of carrot cells with oligogalacturonides activates phytoalexin production involving G-proteins [27]. In addition, yeast elicitation of *Cupressus lusitanica* increases β-thujaplicin levels through the jasmonate signalling pathway stimulated by H_2O_2 generation or oxidative bursts that induce lipoxygenase activity, a key enzyme in jasmonate biosynthesis. In this case, the yeast elicitor (oligosaccharide) interacts with a G-protein coupled receptor, which in turn switches on ion channels permeable to Ca^{2+}, followed by oxidative burst [28], in agreement with reports indicating that Ca^{2+} influx, activation of phospholipase C, and H_2O_2 production are G-protein dependent responses in plants [29-31]. Moreover, lipoxygenase activity was shown to be dependent on G-proteins and Ca^{+2} signals [28].

Of interest, ethylene transiently up-regulates activity of several small GTP-binding proteins, probably related to the Rab class in *Arabidopsis* leaves [32]. Ethylene-dependent activation of GTP-binding proteins was also reported in peas [32]. Accordingly, expression of antisense *Rab11* in tomato plants reduces fruit softening, a process associated with ethylene [33]. Interestingly, Rab proteins were reported to be involved in vesicle trafficking [34]. Other roles for these small GTP-binding proteins have been reported by several authors. Li and collaborators showed that Rop proteins, a Rho subfamily found only in plants, are involved in the regulation of a wide range of developmental events [35], particularly cytoskeleton organization in growth of the tip of pollen tube in mung bean [36]. Rop proteins were also involved in elicitor-induced ROS production in soybean cell cultures. Interestingly, during the oxidative burst, this Rop protein translocated to the microsomal membrane. Similarly, Rac2 was reported to mediate the elicitor-induced oxidative burst in tobacco cells [37].

4.2. cAMP messenger system: One of the second messengers accumulated in response to elicitation is cyclic AMP (cAMP). Information regarding the adenylyl cyclase/cAMP/PKA signalling cascade is slender in the plant kingdom. Adenylyl cyclase activity was found in plants [38]. In addition, a protein homologous to fungal adenylyl cyclase was isolated from maize pollen. This protein, named PsiP (pollen-signalling protein) demonstrated adenyl cyclase activity when transfected to *Escherichia coli*. Of interest, inhibition of PSiP by antisense assays disturbs cAMP-dependent apical growth [39]. There is also lack of information regarding the downstream effector for cyclic nucleotides presence, PKA. Nevertheless, some biochemical evidence for cyclic nucleotide-responsive kinases related proteins has been reported [38]. Also, homologue sequences to those encoding the catalytic subunit of mammalian PKA can be found in the *Arabidopsis* genome. However, functional information for regulatory domains capable of binding cAMP is still lacking. Despite these facts, evidence demonstrating cAMP-induced accumulation of phytoalexins has been reported. Zhao and collaborators showed that β-thujaplicin accumulation in *Cupressus lusitanica* cells depends on cAMP levels. Moreover, they found that signalling through the cAMP pathway can negatively affect ethylene biosynthesis or signalling [28]. The mechanism by which cAMP decreased ethylene production is not fully understood. A possible explanation may be related to the cyclic nucleotide-gated channels (CNGCs), which are novel plant ion channels activated by cyclic nucleotides and

permeable to monovalent and divalent cations, such as Ca^{2+} [40]. In plants, Ca^{2+}-permeable CNGCs can probably act at key sites allowing Ca^{2+} and cyclic nucleotide cross-talking. Other plant species also accumulate phytoalexins in response to cAMP, such as carrot [41], lucerne [42] and French bean [43]. Also, enhanced accumulation of phytoalexins was achieved in the absence of elicitors by treatment with cAMP analogues, such as dibutyryl-cAMP. Altogether, this evidence shows that the adenyl cyclase/cAMP/PKA pathway is implicated in elicitation responses.

4.3 IP_3 and DAG messenger systems: Several studies performed on various plant species have contributed to our knowledge of the plant PLC/IP_3-DAG/PKC signalling pathway [44,45]. Pertinent to this paper, production of reactive oxygen species (ROS) and phytoalexin biosynthesis after elicitation of rice cell cultures with *N*-acetylchitooligosaccharide was associated with the activation of phospholipase C (PLC) and phospholipase D (PLD) [46]. Moreover, the enzymatic products of these phospholipases, diacylglycerol (DG) and phosphatidic acid (PA), respectively, could induce ROS generation in the absence of the elicitor, but not phytoalexin biosynthesis. Chitosan effects on anthraquinone production were also shown to be mediated by PLC in *Rubia tinctorum* cells. Thus, anthraquinone synthesis was reduced after treatment with phospholipase C inhibitors neomycin and U-73122 [47]. A polyphosphoinositide- PLC has been cloned in *Arabidopsis* named *AtPLC1*, which is expressed at very low levels under normal conditions, but it is induced by dehydration, salinity and low temperature conditions [45]. Additionally, three distinct partial cDNAs named pVr-PLC1, pVr-PLC2, and pVr-PLC3 encoding isoforms of putative PI-PLC were identified in *Vigna radiata* L. Particularly, the *Vr-PLC3* mRNA level was very low under standard growth conditions, but was rapidly induced after environmental stress [48]. Of relevance, transgenic expression of PI-PLC in maize led to improved drought tolerance of the transgenic maize plants when compared with the wild type ones [49]. This raises the possibility of modulating PI-PLC expression by elicitation in order to increase production of secondary metabolites.

There is evidence correlating IP_3 release to elicitation, as shown for the polygalacturonic acid-induced oxidative burst in soybean cells [44]. IP_3 has also been involved in the accumulation of pisatin in *Pisum sativum* after elicitation with the pathogen *Mycosphaerella pinodes* [50]. Other examples are 6-methoxymellein accumulation in carrot cells [51], anthraquinones in *R. tinctorum* [47], medicarpin in lucerne cell culture [52], β-thujaplicin in the Mexican cypress [53] and synthesis of scoparone in lemon seedlings in the hypersensitive response against *Alternaria alternata*, although in this case IP_3 release was independent of G protein activation [54].

Regarding the role of DAG in plants, a significant increase in the levels of this messenger was observed after elicitation of *R. tinctorum* cells with chitosan [47]. Accordingly, phosphatidic acid accumulation via DAG phosphorylation by DAG kinase is an early response in the Cf-4/Avr4 interaction in *N. tabacum* [55].

There is scarce information on the existence of PKC in plants and its function in elicitation. Nevertheless, PKC homologous proteins have been identified in plants whose kinase activity depends on the presence of DAG. These PKC-like proteins have been involved in elicitor responses like chitosan-induced anthraquinone production in *R. tinctorum* cells [56] and in *Nicotiana tabacum*, responses to either ergosterol or cryptogein [57].

Altogether, this evidence points out the importance of the PLC/IP_3 -DAG/PKC messenger pathway in elicitation and its role in secondary metabolite production.

4.4 Ca^{2+} messenger system: It is widely accepted that early events in elicitation induce calcium influx in the plant cell resulting in a transient increase of intracellular Ca^{2+} concentration from 50–100 nM at rest to 1–5 μM within 5 min after elicitation [58,59]. Ca^{2+} signals are generated through the opening of Ca^{2+} permeable channels from a compartment in which the ion is present at relatively high concentration (from outside the cell or from an intracellular store). Perception of Ca^{2+} signals occurs through their binding to calcium sensors. One of these sensors is calmodulin, which undergoes conformational changes after binding of the cation allowing calmodulin to modulate a downstream target. Another sensor is the Ca^{2+}-dependent protein kinase (CDPK), which is switched on after binding Ca^{2+} [60].

Several studies have demonstrated that Ca^{2+} signals can mediate responses to different stimuli by differing in amplitude, frequency, duration and intracellular localization of the increase in its

concentration [60]. Elicitation of *Nicotiana plumbaginifolia* cell cultures with cryptogein and oligogalacturonides generates specific calcium signals for each compound. When treating with oligosaccharides, a biphasic calcium increase was observed; the first peak was induced by the influx of extracellular Ca^{2+}, whereas the second pulse was caused by PLC activation and IP_3 -dependent Ca^{2+} release from intracellular stores [61].

Of relevance, abscisic acid has been shown to promote Ca^{2+} influx from outside the cell increasing the cytoplasmic free Ca^{2+} concentration in guard cells [62,63]. Abscisic acid also stimulates ROS production, which in turn stimulates Ca^{2+}-permeable cation currents in the plasma membrane termed I_{ca} [63]. ROS production in guard cells can also be induced by elicitors such as chitosan and oligo-GalUA leading to stomatal closure [64].

All this evidence endows Ca^{2+} signalling with an important role in elicitation, also being one of the first responses after perception of the elicitor.

4.5 Mitogen-activated protein kinases (MAPKs): MAPKs are major components acting downstream of receptors and which transduce external signals into intracellular responses in all types of eukaryote cells. In plants, MAPKs can be activated by different stimuli, such us pathogen attack, wounding, low temperatures, drought, salinity, UV irradiation, ozone and reactive oxygen species [65] and also mediate signalling of plant hormones like ethylene and auxin [66]. Upon activation, MAPKs are able to modulate downstream targets. The vast majority of defined substrates for MAPK are transcription factors. However, MAPKs can phosphorylate many other substrates, including different protein kinases, phospholipases, and proteins from the cytoskeleton [5].

Numerous genes encoding MAP kinases and other components of MAPK signal transduction pathways have been identified in plants. All MAPKs must be phosphorylated on both a tyrosine residue at position 215 and on a threonine residue in position 213 for their activation. This threonine-x-tyrosine (TXY) sequence is located in the kinase subdomain VIII [67]. Until today, 20 different MAPKs have been identified in the *Arabidopsis thaliana* genome. Both biotic and abiotic factors lead to MAPK activation in plants. Among the first ones we can mention chitosan activation of MAPK in *Rubia tinctorum* cells [68] or the Myelin Basic Protein (MBP) in *Lycopersicum esculentum* [69]. In *Nicotiana tabacum*, elicitation with cryptogein and harpin activates Salicylic Acid Inducible Protein Kinase (SIPK) and Harpin Activated Protein Kinase (HAPK), respectively [70,71]. MAPK pathways have been implicated in signal transduction of many environmentally aggressive stimuli, such as wind, cold, UV irradiation and wounding. *Arabidopsis* plants exposed to low temperature or salinity stress show transient increases in transcript levels of the genes encoding MEK kinase ATMEKK1, MAP kinase ATMPK3 and also ATPK1, a p90 ribosomal S6 kinase [72]. Also, in tobacco, wounding leads to activation of WIPK [73], a wound-induced protein MAP kinase, while in *L. esculentum* the same stimulus causes MBP activation [69]. It was reported that chitin strongly activates the MAP kinases SIMK, MMK2, MMK3 and to a lesser extent SAMK in alfalfa [74]. These observations suggest that MAPKs are not simply activated or deactivated, but instead they exhibit different degrees of activation. Also, the same pathways may be modulated by different elicitors, as can be seen for SIMK that can be activated by treatment with ergosterol, chitin or β-glucan [74]. The authors raise the possibility that after elicitation, a branching into several pathways must take place, probably at the point where MAPKKKs act, since they can regulate diverse MAPKKs that in turn activate distinct MAPKs.

Activated MAPK generally translocates to the nucleus, where it affects specific gene expression. In turn, MAPK phosphorylates transcription factors resulting in increased expression of genes encoding enzymes that regulate key steps in the synthetic routes of secondary metabolites, including phytoalexins [5,75]. It was shown that MAPKs play a role in ethylene signal transduction. CTR1, a downstream target of the ethylene receptor 1 (ETR1), which is an homologue of members of the Raf family, which negatively regulates the MAPKK SIMKK and the MAPKs SIMK/MMK3 of *Medicago sativa*, and MPK6/13 of *Arabidopsis* [76]. Ethylene inactivates ETR1 and CTR1 and consequently activating SIMKK and in turn ethylene-responsive gene expression. Another example is MAP kinase 4 (MPK4), which functions as a regulator in stress responses, since it is required for activation of jasmonate-dependent defense gene expression [77]. Of interest, a yeast two-hybrid screening assay revealed that the MPK4 substrate, MKS1 interacts with WRKY25 and WRKY33, two members of the WRKY transcription factors. MKS1 may therefore

contribute to MPK4-regulated defense activation by coupling the kinase to specific WRKY transcription factors [77]. Phosphorylation events do not only modulate activity of proteins involved in transcriptional regulation. Thus, in French bean and *Arabidopsis*, phenylalanine ammonia lyase (PAL) is phosphorylated by certain CDPKs after elicitation [78,79].

Although there is an overwhelming amount of evidence that relates MAPK cascades with the elicitation process, clearly further studies are needed in order to provide information on how the signals triggered by elicitor interaction with its receptor are transmitted into MAPK pathways and how they modulate secondary metabolism. Such information will be useful for the purpose of maneuvering MAPK cascades to improve production of secondary metabolites endowed with commercial interest.

5. Jasmonic acid, Ethylene and Abscisic Acid Elicitor-induced Pathways

As reported by many authors, elicitor-induced accumulation of secondary metabolites in plants requires activation and interaction of an endogenous set of signal molecules. These signal molecules can be grouped in major elicitor-induced pathways, which comprise jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). Salicylic acid-dependent elicitor signaling has been less studied and it will not be discussed here [80].

5.1 Jasmonic acid pathway: Jasmonic acid and its derivatives, collectively called jasmonates, are small fatty acid derivatives which are synthesized via the octadecanoid pathway [81]. Jasmonic acid acts as a precursor for the production of jasmonate in response to elicitation, wounding inflicted by herbivores, pathogen invasion, certain symbiotic events and reproduction. Several studies have shown that exogenous application of jasmonates to either plant cell suspension cultures or intact plants efficiently stimulates biosynthesis of secondary metabolites [82,83]. Moreover, many elicitors induce jasmonic acid production as an early response [83-85], which in turn activates the expression of secondary metabolite biosynthetic genes, such as those for indole alkaloids in *Charanthus roseus* [86], phytoalexin in rice [13], indole glucose inolates in *Arabidopsis* [87] and β-thujaplicin in Mexican cypress cell culture [88,89]. Of interest, *Arabidopsis* plants that lack jasmonic acid perception or biosynthesis are unable to initiate appropriate defense-related generation of secondary metabolites [90,91].

Regarding the mechanisms of jasmonate signaling in plant cells, most efforts have been focused on the induction of plant defense responses [83]. A large number of elicitor-responsive elements have been identified allowing the characterization of their cognate binding factors. Examples of this are the W-boxes in the promoter of the parsley *PR1-1* gene [92] and the tobacco chitinase gene *CH50* [93]. Also, the presence of a novel jasmonate and elicitor-responsive element containing a GCC motif in the terpenoid indole alkaloid biosynthetic gene strictosidine synthase in Madagascar periwinkle (*Catharantus roseus*) has been reported [94]. Strictosidine synthase is a key enzyme in alkaloid biosynthesis, which catalyses the condensation of tryptamine with secologanin to form strictosidine. Expression of the strictosidine synthase gene is induced upon elicitation and by methyl jasmonate (MeJA), and elicitor responsiveness depends on jasmonic acid as a secondary signal, as reported by Menke *et al*. [94]. These authors found that the octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA2) proteins bind in a sequence-specific manner to the jasmonate- and elicitor-responsive elements. The role of ORCA2 transcription factors in JA signaling will be discussed in detail in section 6.

Several reports showed that elicitation of *Taxus chinesis* cell cultures activated gene transcription and/or enzyme activity of geranylgeranyl diphosphate synthase (GGPPs) and taxadiene synthase (TS), two important enzymes in the taxoid biosynthetic pathway [95,96]. Recently, a novel synthetic jasmonate derivative, 2-hydroxyethyl jasmonate (HEJ), was shown to have very powerful stimulating effects on the biosynthesis of taxuyunnanine C by *T.chinensis* cells [97,98]. Taxuyunnanine C is a physiologically active substance which has neuron growth factor enhancing activity used for the treatment of Alzheimer's disease. Of relevance, a publication from Zhong *et al.* brings light into the molecular events that lead to modifications in gene expression which regulate secondary metabolite production in jasmonate-induced *T. chinensis* cell cultures [99]. Upon elicitation with HEJ, induction of both jasmonic acid and H_2O_2 took place. Jasmonic acid signaling activated by elicitation with HEJ provoked transcription of GGPPs and TS suggesting a relationship between early (defense signal response) and late (gene activation and secondary

metabolite biosynthesis) reactions of cells after elicitation. Elicitor stimulated JA production in *T. chinensis* cells was a prerequisite for expression of GGPPs and TS genes and consequently enhanced taxuyunnanine C accumulation. Of interest, H_2O_2 was produced after elicitation [88], but no valid evidence was reported regarding whether or not the jasmonate-induced H_2O_2 production was involved in taxuyunnanine C accumulation.

Of relevance, new research brought light into JA signaling and how this cascade promotes nuclear events after elicitation. Protein Coronatine Insentive 1 (COI1) is required for the perception of jasmonate [100], resistance to insect herbivory, and resistance to pathogens [101,102]. In addition, COI1 is also required for transcription of several genes induced by JA or by wounding [101]. This protein forms part of an enzyme complex called SCF^{COI1} and acts together with one of several JAZ proteins, which are normally bound to transcription factors such us MYC2 [103], a gene transcription factor involved in jasmonate signaling in *Arabidopsis,* inhibiting their activity. In JA signaling, jasmonic acid is enzymatically attached to molecules such as l-isoleucine, producing an active ligand, jasmonoyl–isoleucine (JA–Ile) [104], which stabilizes the COI1–JAZ complex. Then, SCF^{COI1} probably tags the captive JAZ proteins with ubiquitin, condemning them to rapid destruction. After JAZ is destroyed, the transcription factors are released allowing transcription of genes that produce proteins involved in synthesis of secondary metabolites and plant defense. A substantial amount of JA regulated transcription is COI1-dependent, supporting the idea that COI1 acts as a principal control of JA signaling [105]. It seems that COI1 is not only required for expression of genes induced by JA wounding, but it is also required for repression of genes whose expression was suppressed by JA and wounding, suggesting a pivotal role for COI1 in wound- and JA signaling [105].

5.2 Ethylene pathway: Ethylene is another well-known plant hormone involved in a wide range of plant processes such us development and defense responses. The key step in ethylene biosynthesis is catalyzed by 1-aminocyclopropane-1 carboxylic acid (ACC). ACC is encoded by a multigene family and is expressed in response to small molecule elicitors, environmental changes, hormonal factors (mainly auxin), fruit ripening and wounding [106-108]. Unlike JA, only in a few cases has ethylene been shown to induce secondary metabolism in response to elicitation, such us ethylene-induced anthocyanin production in grapevine [109,110]. In *Cupressus lusitanica* cell cultures, ethylene induced β-thujaplicin, but such an effect was dependent on JA production [111]. Also, ethylene concentration in culture media is important for secondary metabolite production, being effective at low concentrations, whereas at high levels it can inhibit the biosynthesis of secondary metabolites [111,89].

Other examples of ethylene induced activation of secondary metabolism came from the use of ethrel (2-chloroethylphosphonic acid, which breaks down rendering ethylene). Addition of this compound to *Camellia sinensis* cell suspension cultures enhanced production of phenolics and flavans [112]. Additionally, sanguinarine production by suspension cultures of *Papaver somniferum*, berberine in *Thalictrum minus* and taxuyunnanine C in *T. chinensis* could also be enhanced by ethylene production [113-115].

However, when studying anthocyanin and carotenoid accumulation in suspension cultures of *Vaccinium pahalae*, exogenous application of ethrel significantly reduced growth production of these secondary metabolites. Incorporation of $CoCl_2$ or $NiCl_2$ reduced ethylene accumulation and improved accumulation of secondary metabolites [116]. Paclitaxel production in cell suspension cultures of *Taxus chinensis* and *T. yunnanensis* is also negatively regulated by ethylene [117]. Other negative effects of ethylene on secondary metabolism were reviewed by Zhao *et al.* [118].

Finally, it has been reported that crosstalk between JA and ET signaling pathways leads to coordinate expression of multiple repressor- and activator-type transcription factors allowing plants to optimize the production of secondary metabolites [119,120].

Regarding the molecular mechanism by which ethylene regulates secondary metabolism, it was reported that the response to ethylene in *Arabidopsis* requires the Ethylene-Insensitive3 (EIN3) and Ethylene-Insensitive3 Like Protein (EIL) family of nuclear proteins. These proteins bind in a sequence-specific manner to a DNA promoter region known as primary ethylene response element (PERE). A downstream target for EIN3 is the ethylene-response-factor-1 (ERF1), which contains this element (PERE) in its promoter [121]. The ERF family is a large gene family of transcription factors and is part of the AP2/ERF super-family. The ERF family is usually divided into two major subfamilies, the ERF

subfamily and the CBF/ DREB subfamily [122]. The ERF domain was first identified as a conserved motif in four DNA-binding proteins from *Nicotiana tabacum*, known as ethylene-responsive element binding proteins 1, 2, 3, and 4 (EREBP1, 2, 3, and 4) and currently renamed ERF1, 2, 3, and 4. ERF proteins were shown to specifically bind to the GCC box, which is a DNA sequence involved in the ethylene-responsive transcription of genes [123]. The AP2/ERF super-family also includes the AP2 and RAV families [122]. RAV1 and RAV2 were first identified as full-length cDNA encoding proteins that contain a B3-like domain and an AP2/ERF domain in *Arabidopsis* [124]. Genes in the ERF family encode transcriptional regulators with a variety of functions involved in the developmental and physiological processes in plants, as well as various responses to environmental stimuli. The finding of the tobacco ERFs [125] allowed the identification of many proteins implicated in many diverse functions in cellular processes, such as hormonal signal transduction [125], response to biotic and abiotic elicitors [126-128] and adaptations to the environment, particularly increased cuticular wax accumulation as a strategy for drought resistance [129-131]. Up to date, 139 and 122 ERF family genes were identified in *Arabidopsis thaliana* and rice (*Oryza sativa*), respectively [132]. A future approach should address the elucidation of the specific biological function of each of these genes.

5.3 ABA signaling: Abscisic acid (ABA) is one of the major plant hormones and plays a pivotal role in many plant cellular processes. It is involved in the establishment of bud and seed dormancy, growth regulation, leaf senescence and abscission, and stomatal closure [133-136]. It has been also reported that ABA mediates production of secondary metabolites in some plant cell cultures generated by cold or osmotic stresses [137]. In agreement with these observations, increased ABA levels during treatment of *C. roseus* cell suspension cultures with the osmotic stress inducing agent, betaine, resulted in enhanced formation of indole alkaloids [138]. A similar response was observed after exposing *Taxus* sps cell cultures to either ABA or high concentrations of sucrose and mannitol [139]. Inhibition of secondary metabolite production after ABA treatment has also been reported. Thus, it has been shown that ABA decreases the synthesis of saponin in *Panax quinquefolium* hairy roots [140] and shikonin and its derivatives in cultured cells of *Onosma paniculatum* [141]. A description of additional effects of ABA on secondary metabolism may be found in the article of Zhao *et al.* [118].

Of particular importance for the scope of this review, there is evidence that the ABA-mediated response to various environmental stresses depends on the induced expression of a large number of genes, whose products are involved in plant adaptation to changes in its environment [136,142]. Gene expression is modulated through ABA-responsive elements (ABREs) [143]. One class of the ABREs includes elements that share an ACGT consensus sequence that belongs to the larger family of cis-elements known as "G-box" (G/ABREs) [144]. The ACGT sequence can be recognized by plant basic leucine zipper (bZIP) class DNA-binding proteins [145,146]. A number of bZIP proteins are known to interact with the ABREs. EMBP1 (a basic leucine zipper transcription factor from wheat) and TAF1 (a histone acetyltransferase from *Arabidopsis thaliana*) have been isolated based on their *in vitro* binding activity to G/ABREs [142]. Another class of ABREs, known as coupling elements (C/ABREs), shares a CGCGTG consensus sequence [143]. Three coupling elements have been described, CE1, CE3 and DRE (dehydration responsive element). Both classes of ABREs are almost ubiquitous in the promoter regions of ABA responsive genes of both monocotyledonous and dicotyledonous plants [147]. Of relevance, a single copy of ABRE is not sufficient for ABA-mediated induction of transcription, instead multiple ABREs or the combination of an ABRE with a coupling element is needed for the formation of an ABA-responsive complex (ABRC) [148,149].

Although ABRE-binding factors have been known for some time, several observations suggest that other unidentified factors are involved in ABA-regulated gene expression. ABA-induction of rice rab16A and *Arabidopsis* rd29B genes requires *de novo* protein synthesis [150], suggesting the involvement of ABA-inducible factors. *In vivo* binding of ABA-inducible factors has been demonstrated for the maize rab17 gene [151]. Furthermore, it has been well established by genetic studies that different ABA signaling pathways operate in seeds and in vegetative tissues [133], and tissue-specific ABRE-binding activities have also been demonstrated [152]. Taken into consideration, the above findings are in keeping with the overexpression of ABRE-binding transcription factors and consequent activation of these stress-inducible genes to enhance production of secondary metabolites and stress tolerance.

6. Transcriptional Regulation of Secondary Metabolism

Elicitor signal transduction cascades converge on the cell nucleus where they induce changes in the transcriptional rate of target genes involved in the synthesis of secondary metabolites [153,154]. The presence of elicitor, ethylene or jasmonic acid response elements in promoter regions of these genes has been investigated. Such promoter regions are recognized by transcription factors that bind in a sequence-specific manner and recruit the general transcription machinery to stimulate gene expression. There are several types of transcription factors with different DNA binding domains, such as helix-loop-helix, zinc-finger, or basic zipper, c-MYB and basic-Helix-Loop-Helix (bHLH), which determine to which DNA sequence they can bind [155]. The genome of *Arabidopsis thaliana* contains 133 genes encoding transcription factors characterized by a bHLH domain. Although some have been implicated in either the regulation of secondary metabolism or development, for most of them functional data, including DNA-binding target sequence and specificity, is still lacking [156,157]. Regulation of secondary metabolism pathways at the transcriptional level is the principal mechanism to control its end products in response to elicitation, environmental changes or pathogen attack. Such regulation depends on tissue type and/or nature of the signal (elicitors, plant hormones, aggressive stimuli). It must also be taken into consideration that regulation of genes responsible for the synthesis of several plant secondary metabolites in response to elicitation (and various stress conditions as well) is likely to be identical or at least similar to the regulation of genes that are generally regarded as pathogen responsive [158]. Moreover, understanding these signal transduction paths and transcriptional modifications underlying elicitor-induced production of secondary metabolites is quite important for optimizing their commercial use.

One of the most studied models of genetic regulation of secondary metabolism is the phenylpropanoid pathway, responsible for the synthesis of lignins, hydroxycinnamic amides, flavonoids, phytoalexins and pigments, among others [159]. In addition to their roles in the structure and protection of the plant, phenylpropanoids have an important effect on plant qualities such as texture, flavor, color, and processing characteristics. The enzymes chalcone synthase (CHS), phenylalanine ammonia lyase (PAL) and 4-coumarate CoA ligase (4-CL) catalyze key steps in biosynthesis of phenylpropanoids. The genes encoding these enzymes present highly conserved DNA sequences known as G-box, H-box, L-box, and P-box, which can bind members of c-MYB and bHLH transcription factor families in response to elicitors, light, UV irradiation, wounding and other stresses [153,160]. In maize cell suspension cultures, over expression of R and C1 transcription factors leads to an increase in anthocyanin biosynthesis [161]. R shares homology with the bHLH protein encoded by the vertebrate proto-oncogene *c-MYC*, while C1 is a homologue of the proto-oncogene *c-MYB* product. In *Arabidopsis*, a single MYB-type transcription factor (PAP1) was identified, which upon over expression led to plants with intense purple pigmentation throughout development [162].

Another example of transcriptional regulation of secondary metabolism is the biosynthetic pathway of terpenoid indole alkaloids (TIAs). Alkaloids are a source of many natural products with pharmacological importance. Serpentine and ajmalicine are used as tranquilizers. Strictosidine synthase (STR), which catalyses the initial step, is a key enzyme in production of TIAs. Another important enzyme of this metabolic pathway is tryptophan decarboxylase (TDC). TIA biosynthesis depends on tissue and cell specific factors, and it is responsive to fungal elicitor and jasmonates and environmental factors as well [163]. In particular, STR and TDC mRNAs accumulate in response to methyl jasmonate [164,86], indicating that TDC and STR gene expression is controlled by a common JA-responsive element. In agreement with this interpretation, the STR gene contains a Jasmonate and Elicitor-Responsive Element (JERE), which is recognized by Octadecanoid-Responsive Catharanthus AP2-domain protein 2 (ORCA2), mentioned before. ORCA2 is a transcription factor of the AP2/ERF-domain. ORCA2 mRNA accumulates in response to MeJa, which in turn activates STR expression [94]. Studies employing the protein synthesis inhibitor cycloheximide showed that MeJA induces the expression of STR and TDC, revealing that *de novo* synthesis of ORCA is not necessary. Instead, MeJA activates a preexisting ORCA protein [158]. ORCA3, another member of the AP2/ERF-domain TFs family, also binds to JERE element and activates STR expression [165]. MeJA induces ORCA3, followed by expression of many enzymes of the TIA biosynthetic pathway [163]. Interestingly, a functional G-box (CACGTG) was also identified in the STR promoter, closely located to the JERE

element [166]. In addition, G-box binding factors of the basic leucine zipper (bZIP) class and MYC-type basic helix-loop-helix (bHLH) have been identified [167,168]. G-boxes have been implicated in MeJA responsiveness of plant genes [144]. In *Catharanthus roseus* cells a new N-terminal truncated protein possessing a typical basic helix-loop-helix domain (bHLH) was identified by a yeast one-hybrid screening [168]. The protein was only able to bind the native G-box (CACcaG or aACGTG) in accordance with members of the B group of bHLH transcription factors.

The plant-specific transcription factors WRKY of the zinc-finger type specifically bind to W-box elements (includes TGAC core sequences), where they play a regulatory role in secondary metabolism associated to stress responses. Several reports have indicated that the W boxes can mediate elicitor and/or pathogen inducible transcription [169]. Rapid accumulation of WRKY1,-3,-4, and-5 transcription factor gene products was observed after elicitation, without the requirement of *de novo* protein synthesis [170]. Moreover, in cultured tobacco cells, the specific binding of transcription factors to TGACC and TGACT sequences was increased after elicitation [93]. WRKY genes are expressed in response to many elicitors, such us the rice blast fungal elicitor, and the signaling molecules jasmonic and salicylic acids [171]. Microarray analysis has identified two chitin oligosaccharide elicitor-induced genes in rice cell suspension cultures, OsWRKY53 and OsWRKY71 [171]. In addition, induction of WRKY-type transcription factors is followed by expression of genes such us chitinase and strictosidine synthase [163]. In agreement with these observations, a putative elicitor response element with two W boxes (CTGACC/T) was identified in the tobacco class I basic chitinase gene CHN48. Upon elicitation with xylanase from *Trichoderma viride,* DNA-binding proteins designated as NtWRKY1, NtWRKY2, and NtWRKY4 were identified [172]. CHN48 gene expression occurred after binding of these three proteins to the W box of the gene. Furthermore, the transactivation of W box-mediated transcription by NtWRKY1 and NtWRKY4 was enhanced in response to elicitor treatment [172], suggesting elicitor induced posttranscriptional activation of these NtWRKYs. Also, the TGAC sequences in the ELRE promoter of tobacco CHN48 gene seem to play a crucial role in the elicitor dependent transcription of genes in tobacco cells. Transcription factors can also act as repressors of secondary metabolite accumulation. Accordingly, knocking out the gene for the MYB-type transcription factor MYB4 in *A. thaliana* favored accumulation of sinapate esters in the leaves [173]. Also, transgenic expression in tobacco of the FaMYB1 protein from strawberry (member of the MYB-type family) led to a reduction in the accumulation of anthocyanin and flavonols, suggesting that in strawberry FaMYB1 repress certain steps in the flavonoid pathway [174].

Concluding remarks

The plant kingdom is a promising reservoir of valuable secondary metabolites. The biosynthesis of many of these natural compounds is part of plant defense mechanisms in response to the attack by insects and pathogens, herbivores or adverse environmental conditions. Over the last decades, elicitation has proven to be a successful strategy for enhancing the yield of many bioactive secondary metabolites of commercial interest that can be applied to both mature plants and cell suspension cultures. Regulation of the activity and synthesis of transcription factors provides a flexible network for stimulation of plant secondary metabolite production. Rigorous genetic control of natural product accumulation in plants can be achieved by over expression of one or a few transcription factors. Even though components of the jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) signaling pathways are identified, it is still ignored how these events are connected and the mechanisms by which elicitor signals are transduced and amplified. Also, cross-talk between JA, ET and ABA signaling is largely unknown. Moreover, further characterization of elicitor-induced molecular processes leading to changes in transcriptional rate of genes encoding key enzymes of secondary metabolism is needed. The full dissection of plant elicitor-induced activation of cell signaling pathways, along with analysis of the DNA sequence of known secondary metabolite genes may help to predict the regulation of other genes Extensive work on such areas will render new insights into the elicitation mechanisms and more effective strategies for overproduction of these plant compounds endowed with practical applications.

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