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Plant Science 172 (2007) 861–875
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Review

Molecular aspects of the early stages of elicitation of secondary metabolites in plants

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Received 7 March 2006; received in revised form 29 December 2006; accepted 16 January 2007
Available online 11 February 2007

Abstract

Plants are a source of commercially important secondary metabolites. Elicitation of plant cells in culture represents a useful biotechnological tool to improve the production of these valuable metabolites. Greater knowledge on the mechanism of elicitation has basic as well as practical implications. This review summarizes molecular information available about the early stages of the elicitation process and the mode of action of elicitors. A description is first provided on the importance of plant secondary metabolism, its induction by elicitation, the elicitor concept and classification, factors affecting elicitation and the mechanisms involved, with a major emphasis on the intracellular transduction systems which mediate the actions of elicitors, namely, elicitor receptors, GTP binding proteins, the Ca^{2+} messenger system and the PI3K, PLC/IP_3-DAG/PKC and adenylyl cyclase/cAMP/PKA pathways, which finally act through mitogen-activated protein kinases (MAPKs) affecting the expression of genes related to the biosynthesis of secondary metabolites. Relevant experimental approaches used to study these topics are also discussed.

Keywords: Elicitation; Signal transduction; Plant secondary metabolism; Biotechnology

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0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2007.01.006

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1. Introduction

1.1. Plants: source of valuable metabolites

Human life depends on plants. In addition to basic nutrients like proteins, fats or carbohydrates, plants are a source of pharmaceuticals, cosmetics, food ingredients, wood, cellulose, agrochemicals, flavors, insecticides and pigments. These compounds that are not essential for survival but confer some advantages to plant cells are called secondary metabolites. Within this context, the plant kingdom has the potential to be the best, non-polluting chemical factory. The chemical industry makes enormous efforts to synthesize these products but with limited success. Therefore, secondary metabolism contributes to the economical importance of plants. However, in spite of the advances achieved in the area of plant biotechnology for production of useful metabolites, the levels obtained do not attain commercial application. Among the numerous plant, valuable metabolites obtained using cultured plant cells (Table 1), only the production of shikonin by *Lithospermum erythrorthizon* cell cultures in Mitsui Petrochemical Industry Co. Ltd. (Japan) [1], Purpurina by *Rubia akane* in Nitto Denko Corp. [2] and taxol by *Taxus cuspidata* in Phyton [3] and Bristol-Myers Squibb Co., gained commercial application.

1.2. Plant secondary metabolism and defense response: elicitor concept

The close relationship between plant secondary metabolism and defense response is widely recognized. Plants respond to attack of pathogens, insects and hervibores or to other biotic and abiotic stresses by activating an array of defense mechanisms including induction of biosynthesis of secondary metabolites as phytoalexins, hypersensitive responses and structural defensive barriers, such as lignin deposition on cell wall, among others [5–8].

Studies on the induction by *Phytophthora megasperma* of phytoalexin accumulation in soybean revealed that small molecules of pathogen origin trigger the same response in the plant as the pathogen itself. These compounds are termed *elicitors* [9]. Later, from investigations on the effects of microbial oligosaccharides on plants, it became evident that elicitors could be used as enhancers of plant secondary metabolism. Actually, the term *elicitors* refers to chemicals from various sources, biotic or abiotic, as well as physical factors, that can trigger a response in living organisms resulting in accumulation of secondary metabolites. Then, elicitors are useful tools for improving the production of plant valuable compounds [10–12]. The aim of this paper is mainly to review information on molecular aspects of the early stages of the elicitation process, i.e. intracellular transduction systems which mediate the actions of biotic and abiotic elicitors on plant secondary metabolic pathways resulting in increased production of useful plant metabolites.

2. Classification of elicitors

Many compounds or stimuli that enhance the production of useable secondary metabolites in plant cultures have been identified. In general, elicitors are classified on the basis of their origin and molecular structure (Table 2). Each type of elicitor according to its characteristics can induce specific responses that depend on the interaction elicitor-plant culture. As indicated in Table 2, elicitors may be biotic or abiotic. The biotic elicitors have biological origin, derived from the pathogen or from the plant itself (sometimes called endogenous elicitor). Biotic compounds can be of defined composition, when their molecular structures are known, or have a complex composition when they comprise several different molecular classes making impossible to define an unique chemical identity. On the other hand, abiotic elicitors have not a biological origin and are grouped in physical factors and

Table 1
Useful secondary metabolites produced by plant cell cultures (reviewed in ref. [4])

Compound	Plant species	Application
Ginsenoside	*Panax ginseng*	Food additive
Anthraquinone	*Rubia tinctorum Morinda citrifolia Rubia akane*	Pigment, food additive, pharmaceuticals, insecticides
Diosgenin	*Dioscorea deltoides*	Pharmaceuticals
Vincristine	*Catharanthus roseus*	Pharmaceuticals
Gingkolides	*Gingko biloba*	Pharmaceuticals
Digoxin	*Digitalis lanata*	Pharmaceuticals
Capsaicin	*Capsicum frutescens*	Food flavour
Crocetin	*Crocus sativa*	Food additives
Taxol	*Taxus cuspidata*	Pharmaceuticals
Imperatorin	*Angelica dahurica*	Pharmaceuticals
Morphine and codeine	*Papaver somniferum*	Pharmaceuticals

Table 2
Classification of elicitors

Elicitors			
Biotic		Abiotic	
Defined composition	Complex composition	Chemical	Physical
Chitosan [14–17]	Fungi homogenate [22]	Sodium orthovanadate [26]	Thermal stress [22]
Alginate [10]	Yeast extract [17,23,24]	Vanadyl sulphate [27]	Osmotic stress [28]
Pectin [18]	Fungi spores [25]	Heavy metal salts [23]	UV irradiation [22,24]
Chitin [17,19,20]			Wounding [29]
Elicitins [21]			

chemical compounds. The classification described above only takes into consideration the nature of the elicitor. But these compounds may be also classified according to the interaction plant-elicitor, into two groups: 'general elicitors' which are able to trigger defense responses both in host and non-host plants and 'race specific elicitors' which induce responses leading to disease resistance only in specific host cultivars, depending on the simultaneous presence of *avirulence* and resistance genes in the pathogen and plant, respectively [13]. A race specific elicitor encoded by an avirulence (*avr*) gene (e.g. *avr*9 and *avr*4 from *Cladosporium fulvum*) present in a particular race of a pathogen will elicit resistance only in a host plant carrying the corresponding resistance (*R*) gene (e.g. *Cf-4* and *Cf-9*, respectively). Thus, the absence of either gene product will lead to disease [30]. In the last years significant advances on the knowledge of these interactions have been made [31–33].

3. Factors which influence elicitation

The effectiveness of elicitation as a tool to enhance the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell. Here, we describe some of the main factors that can affect this interaction and thereby the elicitation response.

3.1. Elicitor specificity

There is evidence that the same elicitor can stimulate secondary metabolism in different cell cultures and, on the other hand, that certain plant cultures are responsive to diverse elicitors. Treatment of a particular culture with different elicitors will result in the accumulation of the same compounds, since these are specific of each plant culture. Although, the class of metabolite depends on the plant species, the kinetics of induction or accumulation levels vary with different elicitors. For example, it has been observed that hairy root cultures of *Brugmansia candida* are elicitated by biotic or abiotic compounds, showing differences in the kinetics of the induction and levels of release of hyoscyamine and scopolamine [23]. Curiously, if one assumes that the elicitor signal is detected by the plant cell via a specific receptor (see Section 5.1), the selectivity of the response of a given plant species for a determined elicitor would depend to a great extent on the presence of such molecular entity and on the transduction pathways that each elicitor activates. More studies are then necessary to confirm this hypothesis. Indeed, investigations have shown that in general the type of metabolites elicited are specific for the plant cell culture and are not dependent on the elicitor class.

3.2. Elicitor concentration and treatment interval

The concentration of elicitor is a factor that strongly affects the intensity of the response and the effective dose, which varies according to the plant species, can only be found empirically. It has been demonstrated that elicitor levels, which exert stimulatory effects in certain plant systems when applied to other ones are devoid of activity, reflecting different sensibilities of the molecular components involved in elicitation. For example, in *R. akane* Nakai cell cultures chitosan induces maximum anthraquinone production at a concentration of 20 mg/l [15], while 200 mg/l of this elicitor is the optimum concentration to improve menthol production by cultured *Mentha piperita* cells [34] and anthraquinone production in *Rubia tinctorum* cell suspensions [35]. Kombrink and Hahlbrock using cultured parsley (*Petroselinum crispum*) cells showed that a phytopathogenic elicitor induced pronounced effects on the formation of coumarin derivatives (phytoalexins) depending on its concentration. Moreover, this response was preceded in all cases by proportional increases in the activities of two enzymes of phenylpropanoid metabolism [36]. In general, two types of dose–response curves have been described [36–38], one which corresponds to a typical saturation profile where overdosage of the elicitor will no affect cell viability and the second type showing a sharp optimum.

Respect to the treatment interval, there are few data available. In general the elicitor is in contact with the system until harvest, but the time required for maximum secondary metabolite accumulation is a characteristic of each plant species and normally is preceded by an increase in activity of the metabolic enzymes involved. For example, treatment of *R. tinctorum* cell cultures with *Pythium aphanidermatum* leads to a doubling of anthraquinone content which is preceded by a large rise in isochorismate synthase activity [39].

These facts point out the importance of determining empirically the optimum conditions of elicitation time and elicitor concentration for each system in particular.

3.3. Culture conditions: growth stage, medium composition, light

Commonly, the literature holds the view that the most adequate moment to add the elicitor is during the exponential phase of growth [35,36] when the enzymatic machinery is in the maximum operative status, the response to the elicitor being, in consequence, more efficiently achieved. Another factor is the presence of growth regulators in the medium, which can markedly affect the elicitation of secondary metabolism. For example, carrot cells cultured without auxin do not respond to elicitation [38]. The production of anthraquinone by cell suspensions of *Morinda citrofolia* is also affected by different growth regulators [40]. Likewise, culture light conditions may also play a significant role, as in jasmonic acid-induced hypericin production in *Hypericum perforatum* L. cells which exhibit higher cell growth and secondary metabolite production when incubated in the dark than under light [41], whereas other studies have reported light stimulation of secondary metabolite synthesis [42].

In view of the variability in elicitation responses due to different factors like those described above, the optimization of medium composition and culture conditions represent an important aspect in elicitation protocols.

4. Effects of elicitation on turnover and storage of secondary metabolites

Elicitors may affect plant secondary metabolism by modulating the rates of biosynthesis, accumulation and/or vacuolar transit, turnover and degradation [43]. It is known that phytoalexin levels can be regulated by various elicitors through one or more of these mechanisms [22]. Rhizosecretion of genistein from *Lupinus luteus* L. is increased by chitosan, KCN and salicylic acid, as a consequence of stimulation of de novo synthesis [44], whereas chitin enhances anthraquinone formation and also its release into the medium by permeabilization of the plasma membrane [45].

Although, our current knowledge on the mode of action of elicitors is related almost exclusively to secondary metabolism, in recent years it has been demonstrated that primary metabolism may also be affected by elicitation. For example, elicitation with methyl jasmonate induces marked changes in central metabolic pathways affecting secondary metabolism in *Medicago truncatula* cell cultures [24]. Also, it has been shown that treatment of poppy cell suspension cultures with a fungal elicitor resulted in the induction of glutathione *S*-transferase (GST) [46]. GSTs are dimeric enzymes that catalyze the conjugation of electrophylic molecules to glutathione (GSH). In plants, these conjugates are sequestered in the vacuole [47]. In addition to catalyzing GSH conjugation reactions, GSTs can function as carriers of auxin and phenylpropanoids and transporters of anthocyanin into the vacuole [48,49]. Within this context, it is feasible that elicitation modulates the expression of molecules of primary metabolism involved in vacuolar transport and thereby regulates the levels of secondary metabolites. Given that transport and storage play a key role in achieving high production of valuable compounds, further knowledge on the factors that control these processes may have biotechnological implications.

4.1. Elicitor-triggered phytoalexin accumulation and excretion

One of the best-studied responses to stress in plants is the biosynthesis of phytoalexins, secondary compounds of low molecular weight with antimicrobial properties. The application of various abiotic or biotic elicitors can trigger the synthesis and accumulation of these compounds [50]. With regard to the elicitor-induced excretion of phytoalexins from the cells into the medium, some authors explain this fact as a consequence of disturbances of cell permeability, osmotic conditions or changes in membrane potential caused by elicitor treatment. On the other hand, the possibility of cellular death induced by elicitation is also considered. In this case, the excretion of metabolites can be explained by leakage or cellular lysis [43]. This hypothesis does not rule out, as mentioned above, the possibility that elicitation may promote transport reactions.

4.2. Elicitor regulation of turnover of constitutive secondary metabolites

Sometimes it is not easy to establish a difference between induction of secondary metabolites by elicitation and increment of constitutive secondary compounds by elicitor action, since elicitors generally affect total secondary metabolism [43]. For example, it is well established that alkaloids can be constitutive or inducible. Likewise, phytoalexins and various other secondary metabolites related or not to their biosynthesis may accumulate by elicitor treatment. Furthermore, complex mixtures of newly synthetized and constitutive compounds have been evidenced upon elicitation [43,51].

5. Molecular mechanism of action of elicitors on secondary metabolism: signal transduction

5.1. Plant elicitor receptors

As mentioned before, elicitors belong to a wide range of different classes of compounds; generally without having a common chemical structure (Table 2) and not all plants are responsive to all of them. Compounds that can elicit the production of a particular metabolite in certain plants, can be inactive in other species. On the other hand, different plant species can be responsive to the same elicitor. In addition, we show below that there also exists elicitor specificity respect to the signal components activated by elicitation. These facts suggest that plants have the ability to recognize a number of structurally distinct molecules as signals and could be explained by the existence of specific receptors for each elicitor class.

Signal perception is the first step of the elicitor signal transduction cascade and, for example, recognition of different stimuli is central to the ability of plants to respond through

activation of kinases, generation of reactive oxygen species, ion fluxes and cytoplasm acidification. Although, the hypothesis that elicitors act by interaction with receptors on the plant cell surface emerged several years ago, after studies which showed the presence of proteins with receptor characteristics in soybean membranes [52,53], recent results have significantly improved our understanding of elicitor perception. As shown in Fig. 1, numerous elicitor binding sites have been identified, and all these putative receptors were localized in plant plasma membranes. Among the different classes of receptors studied, transmembrane receptor-like protein kinases (RLKs) represent one of the most likely categories of receptors implicated in pathogen perception, although the variety of plant RLKs and the large number of them present in the *Arabidopsis* genome suggest that RLKs may be concerned with the perception of a broad range of stimuli [reviewed in 54]. By far the best characterized RLK, employing molecular genetic approaches, is the flagellin receptor, which belongs to a leucine rich repeat (LRR) class [55,56]. Other category of elicitor receptors comprises the plant R-proteins that recognize race specific elicitors encoded by avirulence (*avr*) genes [57]. This interaction between plant R-proteins and *avr* products is highly specific and could explain why some elicitors induce the biosynthesis of phytoalexins while others cannot. A characteristic structural event of these R-proteins is the presence of the nucleotide-binding site and leucine-rich repeat domains (NBS-LRR). NBS-LRR proteins as, for example, Rps2, L6, Rpp5 Prf, among others, have a conserved leucine-rich repeat domain, which may be responsible for recognizing specific *avr* elicitor [58,59]. Even though that a direct receptor-elicitor interaction has been established, the recognition event is often likely to be more intricate [60]. R-proteins may represent an example of interactions with a superior level of complexity, since the *avr* product does not directly interact with a R-protein but at least three players are needed to trigger resistance [61]. Undoubtedly, this primary event in elicitor signal transduction should be an important subject of future investigations. Likewise, there is no information on the role of the lipid environment of plant receptors. It is known, that the membrane framework surrounding receptors in animal cells affects their structures, dynamics and functions. Thus, future studies considering these aspects are likely to reveal that the receptor-elicitor interplay is of superior complexity than the events identified up to present time.

5.2. Intracellular signaling systems involved in elicitation

5.2.1. GTP binding proteins

Although, many details related mainly to the function of plant G-proteins are still unclear, their existence in plants is indisputable [72,73]. Several lines of evidence show that plant G-proteins have similar properties to those found in other organisms. Signal-transducing GTPases in plants include small G-proteins, heterotrimeric G-proteins and, potentially, several exclusive types of GTP-binding proteins that are not members of either of the afore mentioned classes [72]. The *Arabidopsis* genome contains only a single canonical Gα (GPA1) and Gβ (AGB1) subunits and possibly two Gγ subunits [73]. On the other hand, the number of gen copies for small G-proteins is larger [74].

Plant G-proteins have been involved in various cellular processes linked to growth, hormone signaling, development

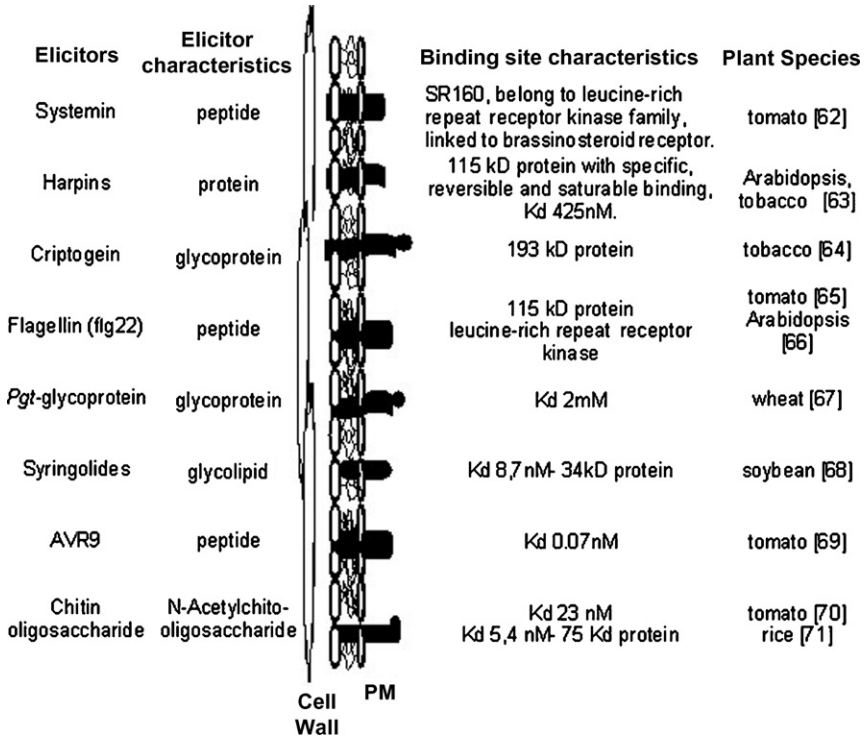

Fig. 1. Scheme indicating elicitor binding sites in various plant cells.

Table 3
Evidences showing the participation of G-proteins in elicitor actions

G-protein activated	Elicitor	Sensitivity		Plant species	Experimental approach	Ref.
		Pertussis toxin	Choleric toxin			
Heterotrimeric and monomeric	Nod factors (1 nM)	(+) 0.5 μg/ml	(+) 2 μg/ml	*Vigna unguiculata L. Walp*	Pharmacological GTP-binding assay [^{35}S]GTPγS overlays WB: anti-Gα_{common} Anti-Rac1 (C-11)	[75]
Heterotrimeric	Race-specific fungal elicitor (0.1–0.35 μg/μl)	–	–	*Lycopersicon esculentum* L.	Pharmacological GDPßS (300 μM) GTPyS (300 μM)	[76]
Heterotrimeric	Chitosan (200 mg/l)	–	(−) 2.5 μg/ml	*R. tinctorum* L.	Pharmacological GDPßS (1 mM) GTPyS (300 μM)	[77]
Heterotrimeric and monomeric?	Oligogalacturonide (5 μg)	–	–	*D. carota* L.	Pharmacological GDPßS (100 μM) GTPyS (100 μM) GTPase activity GTP-binding activity	[78]
NtRac5 (Rac GTPase)	Cryptogein (20 nM)	–	–	*N. tabacum* L.	Genetic procedures WB: RGS-4xHis	[79]
Heterotrimeric	*Cladosporium fulvum* (1.25–1.40 μg/μl)	–	–	*Lycopersicon esculentum* L.	Pharmacological	[80]
Heterotrimeric	YE (1 mg/ml) H_2O_2 (10–24 mM), MeJa (100 μM)	(+) 2 and 4 μg/μl	–	*Cupressus lusitanica*	Pharmacological GTPase activity GTP-binding activity	[81]
Heterotrimeric OsRac1	Sphingolipid (10 μg/ml)	–	–	*Oryza sativa* L.	Genetic procedures	[82]
Heterotrimeric	*V. dahliae* 277 (70 μg/ml)	(−) 100 μg/ml	(+) 24 μg	*Glycine max*	Pharmacological WB: anti-Gα_{common}	[83]
Rop-like GTPase (analogous to Rac)	Hyposmotic shock OGA (7 μg/ml), harpin (40 μg/ml)	–	–	*Glycine max*	[γ-35S] GTP-Binding Genetic procedures WB: AntiRac1, AntiRac2, Anti-Rop	[84]

(+) Sensitive; (−) insensitive; YE: yeast elicitor; MeJa: methyl jasmonate; OGA: oligo-GalUA; WB: Western blot assay.

and defense responses [72,74], and an increasing body of knowledge has involved G-proteins in the response to elicitors. Table 3 summarizes evidences supporting the participation of G-proteins in elicitation mechanisms. This information is generally based on the effect of biochemical agents known to affect G-protein functions in animals, such as non-hydrolysing GTP analogues, mastoparan, suramin, AlF_4^-, melitin, bacterial toxins. However, the application of molecular and genetic approaches has validated the conclusions [82,85]. For example, H_2O_2 production and PR gene expression induced by elicitors are strongly suppressed in rice dwarf1 (d1) mutants lacking a single-copy Gα gene [82]. In addition, with proteome analysis, a probenazole-inducible protein (PBZ1) was detected in wild type, but not in the d1 mutant [86]. Using RNA interference technology, the silencing of a small GTPase, OsRac1, resulted in a strong reduction of protein levels and kinase activation by sphingolipids [87]. There is controversy, however, regarding the use of pertussis toxin in plant systems since all the sequenced Gα-proteins do not contain the PTX-reactive cysteine as in mammals, in spite that plant sensitivity to the toxin has been widely shown [88,89, and references therein]. It is evident that the PTX sensitivity observed responds to non-classical mechanisms and additional studies are then necessary to elucidate them.

Although, many details related to plant G-proteins are still unclear, undoubtedly both heterotrimeric and small G-proteins are involved in the biosynthesis of various plant secondary metabolites.

5.2.2. PLC/IP$_3$-DAG/PKC

Much of our knowledge on the plant PLC/IP$_3$-DAG/PKC cascade was initially derived from investigations on animal systems and although there are a large number of unresolved aspects to be investigated, intriguing similarities have been recently found suggesting a common evolutionary origin. Thus, there are evidences confirming that the PLC/IP$_3$-DAG/PKC pathway occurs in plants [85,90,91–94, and references therein]. Several of these studies showed that this cascade plays a role in the responses of plants to elicitors [95]. Accordingly, the number of elicitors that have been found to activate phospholipase C (PLC) in plants causing polyphosphoinositide turnover and production of the second messengers inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) is increasing. For example, in Arabidopsis, a gene (*AtPLC1*) was cloned that encodes a genuine PI-PLC. *AtPLC1* is expressed at very low levels in plants under normal conditions but is induced to a significant extent under abiotic stimuli, such as dehydration, salinity, and low temperature [90]. Moreover, in *Vigna radiata* L. three distinct partial cDNAs (pVr-PLC1, pVr-PLC2 and pVr-PLC3) have been identified, which encode isoforms of putative PI-PLC, and, in agreement with the preceding observations, the *Vr-PLC3* mRNA level was very low under standard growth conditions but was rapidly induced by environmental stress [96]. Three distinct PI-PLC isoforms were cloned in *Solanum tuberosum* leaves, StPLC1, StPLC2 and StPLC3, which are affected by drought stress in a gene-specific manner [92]. With respect to the participation of this pathway in the modulation of secondary metabolism by elicitors, it has been found that chitosan stimulation of anthraquinone synthesis in *R. tinctorum* could be greatly reduced with the PLC antagonists neomycin and U-73122 [35]. Furthermore, chitosan increased IP$_3$ and DAG levels, but in presence of neomycin the induction of DAG by the elicitor was abolished [97].

A role for IP_3 in releasing intracellular calcium is well established in plants [94,95,98]. Moreover, in Arabidopsis, an accumulation of IP_3 that correlates with calcium mobilization was observed in response to abiotic stress [99]. Likewise, IP_3 signaling is involved in biotic elicitor-induced accumulation of pisatin in pea [100], furanocoumarins in parsley [101], anthraquinones in *R. tinctorum* [97], medicarpin in lucerne cell cultures [102], scoparone in lemon seedlings [103] and β-thujaplicin in the Mexican cypress [104].

In contrast to the IP_3 messenger, the role of DAG in plants is less clear. Nevertheless, a significant increase in DAG levels induced by chitosan was observed in suspension cultures of *R. tinctorum* [97], which could be related to increased PKC in elicitation by this agent [35]. In pea, elicitor-induced DAG seems to be required for phytoalexin accumulation since inhibition of DAG conversion into phosphatidic acid (PA) can potentiate the production of pisatin [105]. It has also been reported that elicitors and mastoparan rapidly down-regulate the levels of DAG derived from phosphatidylcholine (PC) so that the list of established phospholipases in plant signal transduction may be extended to include a PC-PLC [106]. Likewise, PA accumulation, via DAG phosphorylation by DAG kinase, is an early response in the Cf-4/Avr4 interaction in *Nicotiana tabacum* [107]. It was also shown that PA is induced by tetraacetylchitotetraose, xylanase and flagellin in suspension-cultured tomato cells [108].

The engagement of PKC, the other member of the pathway here reviewed, in elicitor action is more difficult to establish since it is usually accepted that the role of PKC in plants is fulfilled by CDPKs [109,110]. Nevertheless, PKC homologous genes have been identified in other plant species, and full purification of a plant PKC-like protein and its partial characterization have been described. Thus, this enzyme exhibited all the biochemical properties of conventional PKC: activation by phorbol esters, phosphorylation of the specific sustrate histone H1, phosphatidylserine and calcium dependence [111]. Using selective activators and inhibitors, the potential biological role of PKC in plants and its relation to elicitor mechanisms is beginning to emerge [35,111–113]. Although the use of these synthetic compounds could be questioned since they have been scarcely tested in plants and in general are considered specific for animals, due to the structural differences between PKC and CDPK and in cofactor requirements, their use allow to distinguish between both enzymes. For example, calphostin C, which interacts with the PKC regulatory domain by competing at the binding site of diacylglycerol (DAG) and phorbol esters, is devoid of inhibitory activity on CDPK as it does not require DAG for its activation [114,115]. Likewise, it has been shown that diolein and PMA are unable to affect the activity of the CDPK ZmCPKp54. In addition, bisindolylmaleimide, a specific PKC inhibitor, is inactive on ZmCPKp54 [114]. Congruent with a role of PKC in elicitor effects on secondary metabolism, it has been observed that the phorbol ester PMA activates the chitosan-induced anthraquinone production by *R. tinctorum* whereas the inactive analogue α-PMA is without effects [35].

Additional information is necessary to fully explain the role of the PLC/IP_3-DAG/PKC system in elicitation and the significance of enzyme isoform specificity in certain cases.

5.2.3. Adenylyl cyclase/cAMP/PKA

In contrast to the animal kingdom in which the adenylyl cyclase/cAMP/PKA pathway is well characterized, in plants the information related to this messenger system is scarce, in part due to experimental limitations. For example, cAMP concentrations in plants are generally lower than in animals and the application of standard methods of detection can be unsuccessful [116]. Nevertheless, cAMP levels have been shown to augment in response to elicitor treatment and mediate the stimulation of phytoalexin biosynthesis in several species, such as carrot [117], lucerne [118], French bean [119] and Mexican cypress cell cultures [120].

Kinases would be expected to be a main target for cAMP action based on comparison to animal systems in which PKA assumes this role. Although several groups have cloned genes with homology to PKA [121–123], DNA sequence homology is not enough to define protein function. At the moment, only the partial purification of a protein kinase from petunia, which phosphorylates Kemptide (LRRASLG), a synthetic substrate for PKA, has been reported [124].

With respect to adenylyl cyclase (AC), source of cAMP, the information status is similar to PKA and cAMP, perhaps due to the diversity of known ACs, which precludes a homology search. Moreover, a gene for this enzyme has been not cloned in plants.

In spite of incomplete characterization, the adenylyl cyclase/cAMP/PKA pathway has been implicated in the elicitation process in *Cupressus lusitanica* and Arabidopsis [120,125]. However, pharmacological evidences have shown that the AC/cAMP/PKA cascade does not contribute significantly to the elicitation mechanism triggered by chitosan in *R. tinctorum* [14]. Once more, these facts point out the importance of elucidating the molecular basis of elicitor specificity in the stimulation of the production of plant secondary metabolites.

Multiple new tools are now available for conclusive investigations on whether cAMP acts as a second messenger in plants, but a convenient alternative could be the systems that respond to elicitation through an enhancement of cAMP generation allowing to overcome the difficulties originated by the lower concentrations observed in plants.

5.2.4. Ca^{2+} messenger system

Calcium is an ubiquitous signal in plants which mediates the regulation of many cellular processes by different stimuli, among them, elicitation. There is evidence that the action of many elicitors involves changes in the intracellular calcium status. This concept was first developed by the use of pharmacological agents known to be selective in mammals, although their specificity has not been always tested in plants. However, further support has been more recently obtained by application of the membrane patch-clamp technique and procedures for measurement of plant intracellular Ca^{2+} concentration [$^{45}Ca^{2+}$], using Ca^{2+}-sensitive dyes [126; reviewed in 127,128].

Moreover, it is recognized that elicitor-induced calcium influx is an early response of plant cells, generally resulting in changes from the Ca^{2+} resting level of 50–100 nM to 1–5 μM, within 5 min after elicitor treatment [128]. This fact raises several questions concerning how different elicitors can develop distinct responses mediated by the same single messenger. Several studies have demonstrated that Ca^{2+} signals triggered by different stimuli differ in amplitude, frequency, duration and intracellular localization [reviewed in 129]. For example, elicitation of Nicotiana *plumbaginifolia* cultures with cryptogein and oligogalacturonides generates specific calcium signals for each compound exhibiting different lag and peak times, intensity, and duration [130]. In the case of treatment with oligosaccharides, a biphasic calcium increase was shown, the first peak 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 calcium stores [130]. Also, the exposure of tobacco seedlings to crude elicitor preparations from yeast and *Gliocladium deliquescens* induced an increase in $[Ca^{2+}]_I$, this increase being different to calcium responses triggered by abiotic stimuli, such as cold shock and wind stimulation [131]. A further mechanism by which Ca^{2+} signals mediate specific effects involves the origin/localization of the signal to a determined cellular region. The scheme of Fig. 2 illustrates the localization of specific Ca^{2+} signals generated by various elicitors.

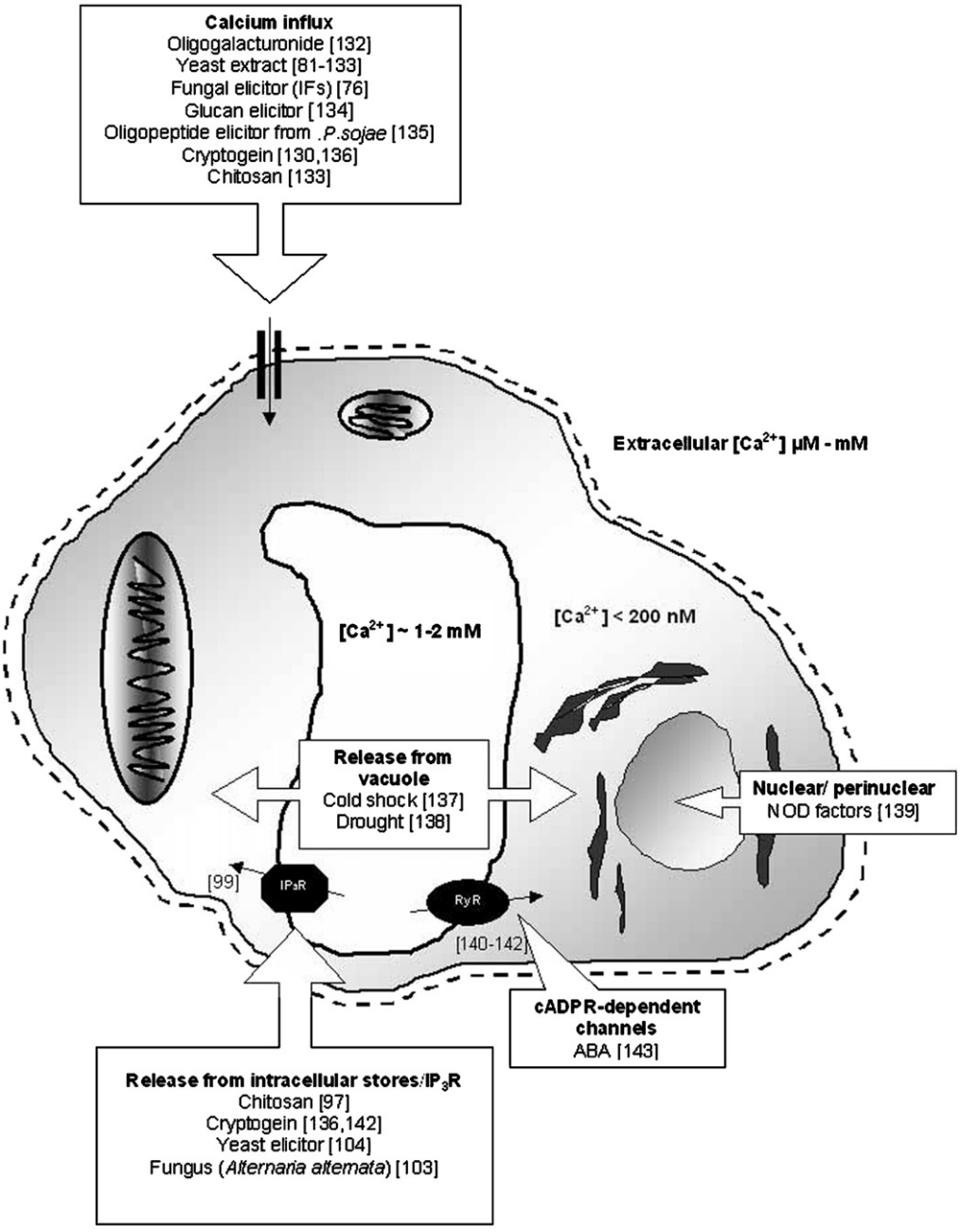

Fig. 2. Scheme showing the origins and locations of calcium signals in response to diverse stimuli.

The data here summarized strongly indicate then that Ca^{2+} plays a crucial role mediating elicitor actions, Ca^{2+} spiking being one of the earliest events that regulates almost all pathways involved in the elicitation process.

5.2.5. PI3K. PI3K-III type (yeast Vps34-like)

Several distinct PtdIns 3-kinase isoforms are known to exist in eukaryotic cells and have been divided into four families based on sequence homology and their preferred inositol lipid substrates. Only one family (type III, that acts on phosphatidylinositol exclusively) has been found in plants, namely the PtdIns-specific Vps34p-related 3-kinases [144,145].

Recently, we demonstrated that phosphoinositide 3-kinase also plays a role in elicitation-mediated secondary metabolite production. Thus, the increase in the synthesis of anthraquinone in *R. tinctorum* L. induced by chitosan is mediated by PI3K activity [97], which in turn stimulates MAPK (see next section). In addition, cloning of *BnVPS34*, a gene that encodes for phosphatidylinositol 3-kinase in *Brassica napus*, has shown that its expression levels are affected by abiotic stress [146].

Although in many aspects the role in plants of PI-3K is imprecise, its activity has been associated with growth, development and membrane trafficking [144]. Curiously, Bunney et al. provide a new perspective on the role of PtdIns 3-kinase in plant cells: their data show that PtdIns 3P is formed in plant nuclei and that PtdIns 3-kinase is localized at active nuclear transcription sites [145]. These findings suggest that the nucleus and nucleolus are among the subcellular locations where at least PI3K and their products exert their functions and demonstrate the complexity of the regulatory network of phosphoinositides. Furthermore, they point out the need of additional investigations, as it may be possible that translocation of class III PtdIns 3-kinase to the nucleus plays a role in regulating transcription together or not with MAPK, in response to elicitation in plants.

5.2.6. Mitogen-activated protein kinases (MAPKs): SIMK, SAMK, SIPK, WIPK. Importance of TEY domain. Recognition of an elicitation stimulus leads to the activation of specific genes

Near 20 different MAPKs have been identified in the *Arabidopsis thaliana* genome. Probably other plant species possess a similar number of MAPKs. Based on phylogenetic analysis, plant MAPKs can be classified into six subfamilies. All plant MAPKs have the evolutionary-conserved Thr–Glu–Tyr activation motif or TEY domain, except members of subfamily V. The TEY domain is located between subdomains VII and VIII of the catalytic core [147]. Phosphorylation of both threonine and tyrosine in this motif is required for full activation of MAPKs [148,149]. Various studies mentioned here, involving plant MAPKs in elicitor signaling, have used antibodies developed for investigations with animal cells, which specifically detect the active phosphorylated forms of MAPK. Thus, the existence of TEY domain in plants validates their use.

MAPK cascades are major components downstream of receptors/sensors that transduce external signals into intracellular

Table 4
Evidence for MAPK involvement in elicitation

Elicitor	System	Plant species	Signaling components/effects involved (upstream)	Signaling components/effects involved (downstream)	Protein/gene	Mapk involved	MW (kDa)	Ref.
Chitosan	Plants	*Lycopersicon esculentum*	–	*DEF1* gene product	P^{48}	MBP	48	[150]
Chitosan	Suspension culture	*Rubia tinctorum*	PLC, DAG, IP_3, PI_3K, PKC, Ca^{2+}	Translocation to nucleus	–	MAPK	~42–44	[14]
Parasiticein Cryptogein	Suspension culture	*Nicotiana Tabacum*	–	Media alkalization *PAL* gene expression	P^{48}	SIPK	48	[151]
Harpin	Leaves	*Nicotiana Tabacum*	–	–	P^{49}	HAPK	49	[152]
Cold	In vitro grown plants	*Arabidopsis thaliana*	–	–	*ATMPK3*	ATMPK3	–	[153]
Wounding	Plants	*Lycopersicon esculentum*	–	*DEF1* gene product	P^{48}	MBP	48	[150]
Sphingolipid elicitor	Cell culture	*Oryza sativa*	OsRac1	–	*OsMAPK6*	MAPK6	65	[87]
Fungal elicitor	–	*Solanum tuberosum*	–	–	*StMPK1*	StMPK1	51	[154]
Fungal elicitor	Suspension culture	Wheat	Ca^{2+}	–	*WCK-1*	WCK-1	48.2	[155]
YE	Suspension culture	*Medicago sativa* L.	–	–	–	SIMK MMK2 MMK3 SAMK	44–46	[149]
UV	Plants	*C. annuum*	–	–	*MK1 MK2*	MK1 MK2	~43–45	[156]
Wounding	–	Tobacco	–	PLA_2 jasmonic acid synthesis	*WIPK*	WIPK	43	[Review in 157]

MBP: 48-kDa myelin basic protein; WIPK: wound-induced protein kinase; SIPK: salicylic acid inducible protein kinase; HAPK: harpin-activated protein kinase; MAPK: mitogen-activated protein kinase.

responses in all eukaryotes. Plant MAPKs are activated by a variety of biotic and abiotic stimuli, including pathogen attack, wounding, temperature, drought, salinity, osmolarity, UV irradiation, ozone and reactive oxygen species [150]. This activation affects next other pathways or specific genes.

In Table 4, we summarize evidences involving MAPK cascades in elicitor action. The analysis of these data may provide clues on how many signals triggered by different elicitors can be transmitted by MAPK pathways. Judging from the large number of MAPK cascade genes, in the smallest plant genome, it may be possible to assign each of these genes to a specific elicitor-activated MAPK cascade. However, the application of new biochemical and molecular tools, as well as animal studies, suggest that there exists more complexity in the action of MAPKs in elicitation. This is deduced from the fact that a given elicitor generally not only activates a single but several MAPKs. Sometimes, this activation also involves induction at transcriptional, translational or postranslational levels [150,152,158]. Moreover, it has been described [149] that chitin activates strongly SIMK, MMK2, MMK3 and to a lesser extent SAMK in alfalfa, suggesting that the stimulation of MAPK cascades is not an all or none process, but also differences in the degree of duration and activation. These authors have also shown that the same MAPK pathways may be modulated by different elicitors, as, for example, ergosterol, chitin or β-glucan can activate SIMK. They propose that upon elicitor perception, branching into several pathways must take place, probably at the point of MAPKKKs, since they can regulate diverse MAPKKs that in turn activate distinct MAPKs [149]. To sum up, a given elicitor might activate a number of MAPK pathways, and different stimuli can activate the same pathway.

As mentioned above, MAPK activation generally affects specific gene expression. Our laboratory has shown that the final steps in chitosan signaling are mediated by translocation of MAPK to the nucleus [159]. In turn, MAPK phosphorylates transcription factors, which results in increased expression of genes coding for enzymes, which play a critical role in the biosynthetic pathway of secondary metabolites including phytoalexins [160]. In agreement with this interpretation, in *R. tinctorum* infected with *P. aphanidermatum* that contains chitosan, it has been shown that the increase in anthraquinone levels is preceded by an increase in isochorismate synthase transcript and activity levels [39]. Likewise, a rice MAPK termed OsMAPK6 that was activated in suspension cell cultures by a sphingolipid elicitor has been characterized. Silencing of this MAPK caused a reduction of elicitor-induced Phe ammonia-lyase (PAL) [87]. In view of the numerous implications of PAL in secondary metabolism, the regulation of OsMAPK6 represents a tool to manipulate production of desirable metabolites. Thus, depending on the magnitude and kinetics of MAPK activation determined by the factors mentioned above, dissimilar responses can be elicited.

Even though abundant information is available, the complexity of plant MAP kinase signaling implicates that more research on MAPKs in plants is required. The literature reviewed here also underlines the need for solid evidence in order to unequivocally establish the involvement of a given MAP kinase pathway in an elicitation process. There are many false leads used that could be mistakenly followed when a

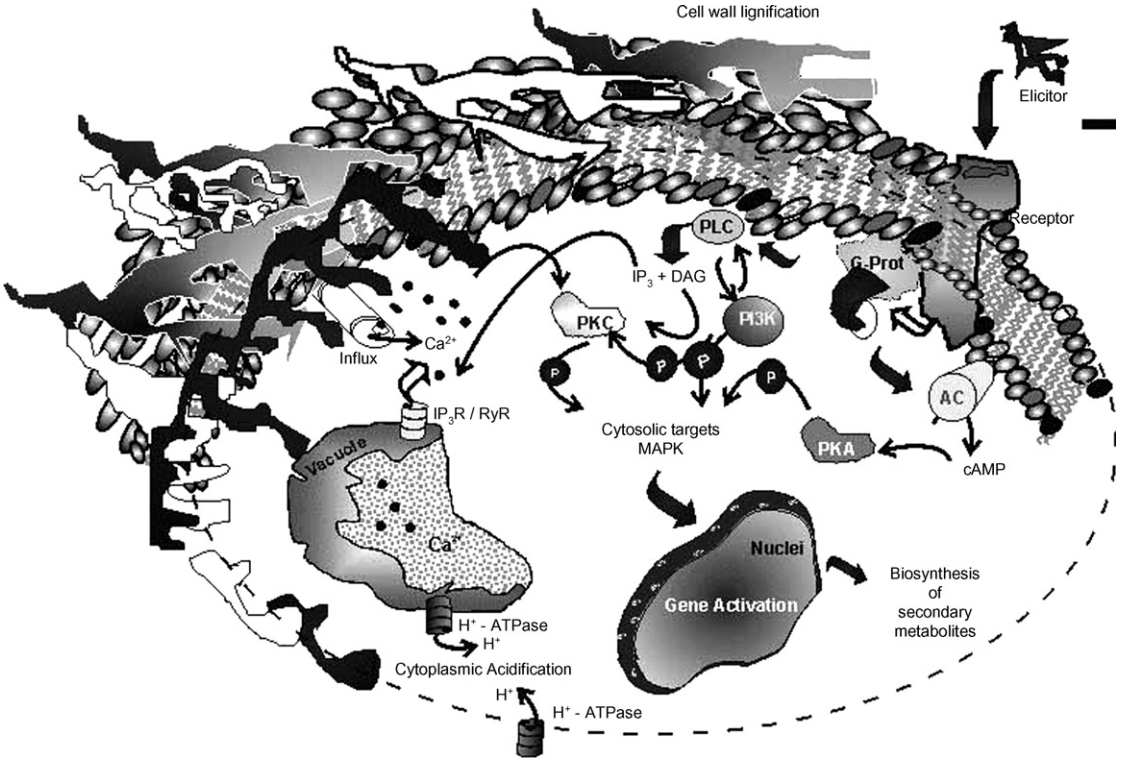

Fig. 3. Key signalling events triggered by the elicitation process.

particular plant MAP kinase action is being monitored, since the protocols and reagents have been generally tested in animals models which could function differently than plants. For example, the identity of the tobacco wounding-activated protein kinase has remained unclear. Discrepancies have been observed between two papers related to activation of such MAPK in response to wounding. Zhang and Klessig presented evidence that wounding activates the 48 kDa tobacco SIPK but not WIPK [151]. In contrast, it has been reported [161] that wounding activates WIPK, suggesting that the myelin basic protein (MBP) substrate in the gel kinase assay used by the former authors might have not been optimal. Also, the production of specific antisera or the generation of transgenic plants bearing individual epitope tagged MAP kinases may be of significance. This would help to eliminate existing uncertainties as to which particular MAP kinase is represented by a MBP kinase activity.

6. Concluding remarks

Over the last decade, increasing knowledge about the elicitation process has brought light into the signaling pathways involved in elicitor responses. On the basis of the information described in the present review, it is possible to delineate the course of the essential events triggered by the elicitor as follows (schematized in Fig. 3):

- Binding of the elicitor to receptors localized in the plasma membrane ensues G-protein activation.
- G-protein-mediated stimulation of adenylyl cyclase (AC) and phospholipase C (PLC).
- Increases of second messengers levels (cAMP, DAG, IP_3) coupled to the activation of their target kinases (PKA, PKC).
- Changes in cytoplasmic Ca^{2+} concentrations: involvement of Ca^{2+} fluxes through plasma membrane or intracellular reservoirs.
- As consequence of the above, rapid activation of protein kinase cascades which induce changes in phosphorylation of MAPKs and in some cases their translocation into the nucleus.
- Activation of transcription of enzymes of synthetic pathways of secondary metabolites.

It is expected that the application of advanced cell biology techniques and molecular genetic approaches will allow the full dissection of plant cell signalling pathways activated in elicitation events. This knowledge might lead to improved strategies for enhancement of the biotechnological production of plant compounds of medical and industrial interest.

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