PHENOLIC COMPOUNDS IN PLANT DISEASE RESISTANCE

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We propose that an important first line in plant defense against infection is provided by the very rapid synthesis of phenolics and their polymerization in the cell wall. This rapid synthesis, which leaves no time for *de novo* enzyme synthesis, is regulated by the extreme pH-dependence of the hydroxylase, catalyzing the formation of caffeoyl-CoA from 4-coumaroyl-CoA. We further propose that elicitor treatment or infection causes rapid membrane changes leading to a decrease in cytoplasmic pH. This decrease would have the effect of activating the hydroxylase.

KEY WORDS: 4-Coumaroyl-CoA 3-hydroxylase; elicitor-treatment; lignification; caffeoyl-CoA 3-O-methyltransferase; caffeic acid derivatives; esculetin; cellular pH-regulation.

INTRODUCTION

Upon contact, phytopathogenic microorganisms induce a number of changes in the metabolism of their target plants. These may include a reduction in total protein synthesis (58), the release of ethylene (12,58), the production of so-called pathogenesis-related proteins (26,71) of lytic enzymes and hydroxyproline-rich glycoproteins (26,38,47), the reinforcement of the cell walls (8), including formation of papillae (1,8,33), and the accumulation of low molecular weight defense chemicals, the phytoalexins (4). These last two changes, in particular, have attracted research attention during recent years, but both the quality and regulation of cell wall modifications occurring during expression of resistance remain as yet poorly understood.

In the case of attack by most biotrophic fungi, changes in the morphology of the host cell wall and plasmalemma determine the mode of interaction. Resistant plants ward off these fungi virtually at the stage of entry. Differences in the formation or

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speed of formation of papillae and collars and in compatible *vs* incompatible interactions have been known for some time, but it has only recently become evident that the expression of resistance may be critically dependent on the quality of the papillae formed in the plant (1;*).

In contrast, some necrotrophic fungi invade the plants irrespective of their genetic predisposition. Inoculation of, for example, safflower and Chinese cabbage with the safflower pathogen *Alternaria carthami* and the potato pathogen *A. solani* resulted in comparable numbers of primary infections (49; K. Mortensen, Regina, Canada, personal communication). Such numbers appear to be an inappropriate measure of the degree of resistance of a given plant. Growth of these fungi in resistant plants usually ceases shortly after infection, with the plant sacrificing a limited number of cells to the invader (29). The actual reason for cell death is, however, unknown. In this kind of interaction, too, the relative speed of formation as well as the quality of cell wall appositions is thought to form the first line of the "complementary defense mechanisms" (6,29) and it may even be critical in halting the growth of the fungus. Here, as well as in the incompatible interactions with biotrophic fungi and viruses, the accumulation of phenolic materials at the infection sites is thought literally to isolate the fungus in the tissue. Several recent reviews have addressed this response (14,15,36,41,51,55,68), suggesting the accumulation of lignin-polymers, but meanwhile it has become more and more apparent that the quality of the induced phenolics has to be questioned.

It has been postulated that, by slowing the penetration of the fungus, the plant gains the time necessary to activate its second line of defense, including the production of phytoalexins in large quantities and their interaction with the invader (4,6). *De novo* synthesis of enzymes involved in the accumulation of phytoalexins in response to fungal attack or elicitor treatment has been confirmed in numerous cases (22,26,31,60) and requires at least 6 to 10 h to reach its maximal rate. If the concept of slowing penetration is correct, the first line of defense in the plant must be triggered by the fungus earlier, and hence it is unlikely to be due entirely to *de novo* transcription and translation. In most plants, the preformed pool of cinnamic acids can also hardly account for the entire phenolic response. The question as to how the initial accumulation of phenolics is regulated remains to be answered.

Only this question and its relationship to the type of induced phenolics are considered here. It is also considered from a point of view different from that presented in previous reviews (1,14,15,26,36,41,51,54,55,60,69). For more detailed information on precursor and enzyme induction studies, the reader is referred to these reviews. The information available on the quality of the lignin induced in resistance expression is first critically evaluated. This is followed by a discussion of recent

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evidence in favor of climatic ester-accumulation. That part of our own work concerning model studies on elicitor-treated plant cell cultures, related to the phenylpropanoid metabolism of cultured cells is summarized and a regulatory mode of action of fungal cell wall components on plant cells will be described.

THE CASE FOR INDUCED LIGNIN

The lignification of plant cells in disease resistance expression has frequently been postulated and the relevant literature has been reviewed by Vance *et al.* (68).

The case for lignification in resistance expression is based primarily on autofluorescence measurements, histochemical staining of infection sites and the fact that carbohydrate polymers within the staining tissue had become resistant to fungal glucanases. Histochemically, the Wiesner or phloroglucinol test is most generally used, whereas the Mäule test, for example, is recommended specifically for syringyl-type lignin and generally causes brown discoloration of other lignins (48). Only the Wiesner test will be considered more closely. It is based on the reaction of phloroglucinol with cinnamaldehyde moieties, which represent a small percentage of the lignin fraction in the cell walls, leading to formation of products with a bright red color (see below). Using this test, intense, red-colored patches around fungal penetration sites have been observed in several plants, in particular in various members of the *Poaceae* (32,55,68) muskmelon (21), carrot (20,28), tobacco (31) and Japanese radish root (31). In the last case, a lignin structure differing from that of normal lignin was postulated (3). The systemic protection of muskmelon (21) and cucumber (10) acquired after previous infection of a single leaf has also been ascribed to a predisposition of the plants to lignify faster in response to wounding and infection (10).

"Lignification" was also induced by elicitor treatment of cucumber (57), wheat (15) and Japanese radish (2), as well as in carrot tissue culture treated with heat-killed conidia of *Botrytis cinerea* (28). An elicitor of lignification in wheat plants has also been isolated from *Puccinia graminis* f.sp. *tritici* (H.J. Reisener, Aachen, personal communication). More recently, Barber and Ride (5) introduced use of a stabilized diazonium salt for lignin determination. This substance couples to cell wall phenolics to form light-dense products in a pale yellow background. When using this assay, cellulase, chitin, chitosan and a crude elicitor fraction from cell walls of *Phytophthora megasperma* f.sp. *glycinea*, as well as the abiotic elicitors mercuric and cadmium chloride, were reported as inducing the lignification of wheat leaves.

The histochemical identification of induced lignin was corroborated in a few instances by monitoring the incorporation of labeled L-phenylalanine (23) and cinnamic acid (10) or the nitrobenzene oxidation of cell wall materials to yield the appropriate aldehydes (10). Furthermore, various enzyme activities implicated in the biosynthesis of lignin (see below) appeared to be induced in plants in response to infection or elicitor treatment. Among these are phenylalanine ammonia-lyase,

cinnamate 4-hydroxylase, 4-coumarate:CoA ligase, cinnamyl alcohol dehydrogenase (22) and peroxidase isoenzymes (9) as well as *ortho*-diphenol O-methyltransferases (31). Their induction pattern, however, and the fact that these enzymes are not specific for lignin synthesis, do not support the theory of early lignification.

THE CASE AGAINST LIGNIFICATION

Protonated cinnamaldehydes formed under the conditions of the Wiesner test are strong electrophilic carbocations which react with phloroglucinol to yield red colored quinonemethides (Fig. 1). It is known that this test is not necessarily specific for lignin since other aldehydes can react mechanistically in the same way, *i.e.*, aldopentoses give rise to stable red colored products (10) while more yellowish products result from various other aldehydes. Recently, a modification of the Wiesner test was suggested (W Heller, München, personal communication) which exploits the peculiar feature of cinnamaldehyde-phloroglucinol adducts to rearrange rapidly to colorless flavenes (Fig. 1). Furthermore, the extraction of lignin from the cell wall material with acetylchloride prior to the assay reduces the possibility of errors due to carbohydrates considerably. Such modifications may be necessary to distinguish cinnamaldehydes from, for example, arabinofuranosyl-moieties, which have been shown to accumulate in response to elicitor treatment (7,47) and appear to stabilize the conformation of hydroxyproline-rich glycoproteins (40,70). Using the modified Wiesner test, lignin formation was not found to be induced in elicitor-treated parsley or soybean cells over a period of 48 h (H. Banholzer, Freiburg, personal communication), although the cells turned dark-brown. Lignification could, however, be induced by changes in the composition of the culture medium. It should be noted here that the hormone regime exerts a strong influence on the lignification response of plant cells (12,22), which makes the interpretation of the respective cell culture experiments difficult. It was also not possible to confirm lignin formation in wheat *plants* infected with *Botrytis cinerea* (A. Heitrich, Freiburg, personal communication). The oxidation of lignin with nitrobenzene and the subsequent determination of aldehydes can likewise be misinterpreted, since the yield of aldehydes in these experiments is usually very low (24,68) and cinnamic esters appear to interfere with the protocol (24,32). On account of these methodological difficulties and the inconsistencies in the results, the proof for *rapid* pathogen-induced formation of lignin in disease resistance is not very convincing at present.

Phenolic compounds which could not be stained with phloroglucinol accumulated in infected sweet potato tissue. These compounds were not related to lignin (67). In contrast, lignin was produced when the tissue was mechanically wounded without infection. Garrod *et al.* (20) demonstrated that, in carrot tissue, various fungi did not stimulate lignification after wounding. Moreover, these authors concluded, in contrast to a previous suggestion by Ride and Pearce (55), that lignification is not rapid enough to play a role in disease resistance. Lignification in infected Japanese radish had been

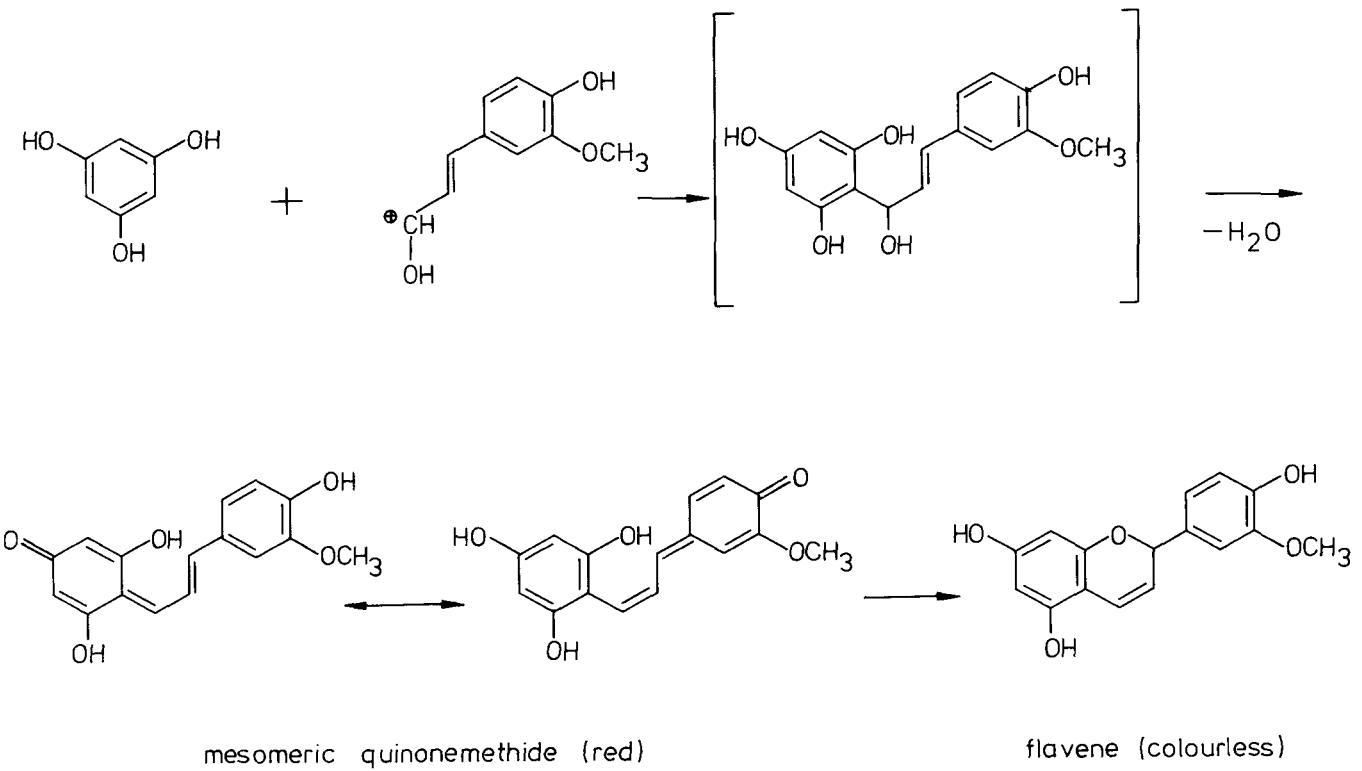


Fig. 1. Reaction mechanism of the Wiesner test for lignin determination. Transient, colored quinonemethides originate by coupling phloroglucinol with cinnamaldehydes under acidic conditions.

earlier suggested by Asada and Matsumoto (2) to represent a delayed resistance response, since phenolics appeared first to be incorporated into the cellulose fraction of cell walls. Upon infection, various potato tissues accumulated phenolics in their cell walls that were not Wiesner-positive, although such cells could be stained with toluidine blue O or acridine orange (25,27). Friend (14) reported that only 4-coumaric acid and ferulic acid, which had presumably been released from carbohydrate esters, were found in alkaline extracts of these potato cell walls. The presence of a feruloyl-β-1,4-galactan was suggested (14). The accumulation of cell wall-bound phenolics and caffeic and ferulic acid esters has also recently been demonstrated in elicitor-treated bean cells (7). Kauss (33) has reported chitosan-induced increases in alkali-soluble wall phenolic compounds from cultured soybean cells occurring concomitantly with the induction of callose synthesis. These increases began about 10 min after addition of chitosan to the cells and continued for a further 30 min (see below). These results support the previous report by Farmer (13) on the accumulation of non-lignin phenolics in elicitor-treated soybean cells.

All of these results appear to suggest that formation of cinnamic esters and related compounds rather than lignin occurs on infection. This interpretation is also compatible with the reported accumulation of 4-hydroxybenzoic, caffeic and ferulic acids parallel to the induction of phenylalanine ammonia-lyase in elicitor-treated carrot cells (38). Ester formation is also consistent with the results of previous precursor and enzyme activity induction studies. The oxidative crosslinking of such esters under the conditions of infection was suggested by Friend (14) and others (68) to proceed in a way analogous to the formation of isodityrosine (17-19).

The biosynthesis of lignin proceeds from L-phenylalanine via 4-coumaric acid and the CoA-esters of 4-coumaric, ferulic and sinapic acids to the corresponding alcohols which supposedly polymerize under the action of a peroxidase (23,24). Whereas the general phenylpropanoid pathway, *i.e.*, the formation of the various CoA esters, is not unique to lignin biosynthesis, an increase in either peroxidase or cinnamyl alcohol dehydrogenase activity in response to infection has been taken as proof of lignin accumulation. However, changes in activity and isozyme pattern of peroxidases appear to occur non-specifically in stressed plants (9) and no lignin specific peroxidase isozyme has been confirmed despite the fact that a likely candidate was suggested (3).

Cinnamyl alcohol dehydrogenase activity, on the other hand, has not been reported as being consistently induced in stressed plant tissues (54). The isozyme pattern of the dehydrogenase, which is known to change with morphogenesis (54), also has not yet been analyzed in elicitor-treated cells or infected plants. Nevertheless, Grand *et al.* (22) recently reported that the dehydrogenase activity in cultured bean cells increased approximately fivefold in response to elicitor treatment, although lignin did not accumulate. Furthermore, no increase in the activity of feruloyl-CoA:NADP oxidoreductase, the enzyme catalyzing the formation of the cinnamyl alcohols, was observed in either parsley or carrot cell cultures (T. Kühnl, Freiburg, personal

communication) in response to elicitor treatment. The accumulation of coniferyl aldehyde and alcohol in flax infected by *Melampsora lini* (34) appears to be exceptional in this context but again does not prove lignin formation, since these intermediates have not been shown to accumulate during regular lignin biosynthesis.

CAFFEIC ACID DERIVATIVES

In all reports a central role in the early expression of disease resistance is ascribed to feruloyl-CoA. Its biosynthesis, however, has not been thoroughly investigated. The formation of caffeic acid, which is thought to be a precursor of feruloyl-CoA from 4-coumaric acid, has been reported to be catalyzed by a non-specific phenolase in spinach beet (72). In extracts from parsley and various other cultured cells, however, we discovered and partially purified a different enzyme activity that is strictly dependent on ascorbic acid and also depends on zinc. Calcium at high concentration (exceeding 1 mM) only partially substituted for zinc, whereas all other divalent cations tested appeared to be inactive. Zinc is not redox-active and hence is unlikely to catalyze the hydroxylation reaction but more probably facilitates enzyme-substrate binding. Detailed analysis of the substrate specificity revealed that the partially purified enzyme preferably catalyzed the formation of *trans*-caffeoyl-CoA from *trans* 4-coumaroyl-CoA rather than the hydroxylation of *trans*-4-coumaric acid. At present, it can not be ruled out that the hydroxylation of either one substrate was catalyzed by a distinct enzyme in the extracts.

Neither the 4-coumaroyl-CoA hydroxylase activity nor the 4-coumaric acid hydroxylase activity was induced upon addition of elicitor to the cells. Both activities are apparently abundant in dark-cultured parsley cells and showed optimal activity at approximately pH 6.5 *in vitro*. This activity decreased sharply both toward pH 7.0 and pH 5.0. At pH 7.2, low enzyme activity (less than 10% of maximum) could be measured and at pH 7.6 the activity was zero. Since the enzyme activities appeared to be localized in the cytoplasm of the cells, a shift in the intracellular pH would be required for their activation *in vivo* (see below).

Dark-grown parsley cells which catalyze the formation of feruloyl-CoA from caffeoyl-CoA also contain significant O-methyltransferase activity (R.E. Kneusel, T. Kühnl and U. Matern , manuscript in preparation). Such an enzyme activity has previously been identified in carrot suspension cultures (U. Koch and T. Kühnl, Freiburg, personal communication). The enzyme appears to be specific for caffeoyl-CoA, since no ferulic acid was detected in control incubations with caffeic acid at low concentration (up to 140 μM). The O-methyltransferase activity of the cells increased approximately five- to tenfold after addition of an elicitor and reached its maximum after approximately 10 to 12 h. In effect, therefore, a pH-signal could trigger rapid production of feruloyl CoA in parsley cells independently of but accelerated by *de novo* induction of enzymes. In this context Vance and Sherwood (69) have reported that formation of "lignified" papillae in reed canarygrass did not require protein

synthesis (see below). No lignin was formed in induced parsley cells according to the Wiesner test and the lignin-specific CoA-ester oxidoreductase activity was not induced. Since feruloyl CoA is also not a substrate for phytoalexin synthesis in parsley (65), its use for ester formation is a likely possibility.

Chlorogenic acid (Fig. 2) accumulates in various plants in response to infection (54). This acid can hardly be classified as a phytoalexin due to its low toxicity. *In vivo,* however, phenolases may convert this compound to the corresponding *ortho*-quinone which is actually responsible for its antimycotic (54,74) and enzyme inhibitory (44) activity. Two pathways have been suggested for the biosynthesis of chlorogenic acid. These involve either the transfer of the caffeoyl residue from caffeoyl CoA to quinic acid or the formation of 4-coumaroylquinic acid and its subsequent hydroxylation (Fig. 2A,B). The latter route most probably predominates *in vivo* although one enzyme appears to catalyze both transferase reactions (54). Friend and collaborators (reviewed under 15) have investigated the role of chlorogenic acid in the disease resistance of potato. Since control tissue often accumulated more chlorogenic acid than infected tissue, they concluded that this compound has no role in the resistance response. Furthermore, in an attempt to distinguish its synthesis from its metabolism, they monitored the hydroxycinnamoyl-CoA(caffeoyl-CoA):quinate hydroxycinnamoyl transferase (CQT) activity (Fig. 2A) by following the formation of caffeoyl-CoA from chlorogenic acid and found it to be higher in the control tissue than in the infected tissue. Care must, however, be taken in the interpretation of such experiments as long as the path of chlorogenic acid metabolism *in vivo* is not known.

In cultured carrot cells 5-O-(4-coumaroyl)quinate and 5-O-(4-coumaroyl)shikimate are formed by the action of CQT and hydroxycinnamoyl-CoA:shikimate hydroxy cinnamoyltransferase (CST), respectively (Fig. 2B,C), although neither product could be recovered from the cells. Instead, the corresponding caffeoyl derivatives accumulated due to the action of a microsomal 3'-hydroxylase (37). Interestingly, irradiation with ultraviolet light decreased the CST activity and concomitantly increased the CQT activity. Addition of a fungal elicitor to the cells affected the enzyme activities in the opposite way (U. Koch and T. Kühnl, Freiburg, personal communication). Whereas chlorogenic acid is known to act as a co-substrate for caffeoyltransferases (54,61,73), no function has yet been ascribed to the shikimate esters. Elicitor-treated parsley cells developed a 4-coumaroyl-CoA:shikimate coumaroyltransferase activity (Fig. 2C) which accepted to a much lesser degree quinate instead of shikimate (T. Kühnl, Freiburg, personal communication). A microsomal 3'-hydroxylase, catalyzing the formation of *trans* 5-O-caffeoylshikimate (30) (Fig. 2C), was induced at the same time. Neither this compound nor chlorogenic acid, however, accumulated in the cell cultures (T. Kühnl, Freiburg, personal communication). An analogous induction pattern in dark-grown parsley cells was observed when the culture medium composition was changed such that lignin formation was induced (H. Banholzer, Freiburg, personal communication).

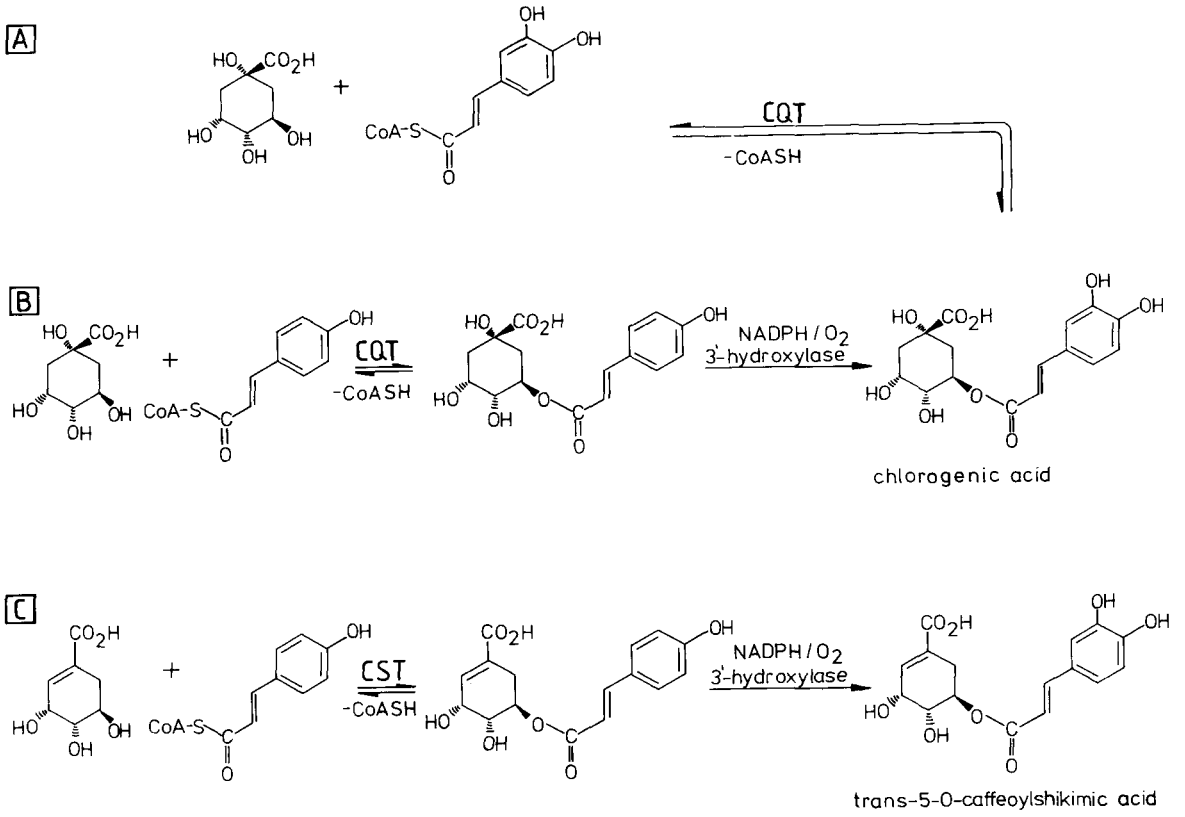
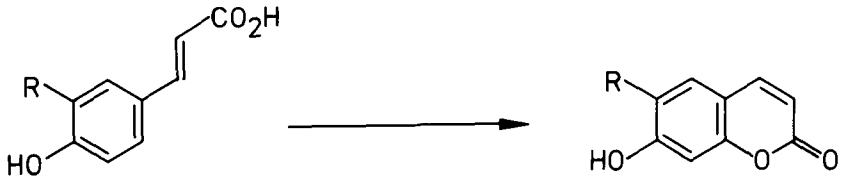


Fig. 2. Biosynthesis of chlorogenic acid (A and B) and 5-O-caffeoylshikimic acid (C) catalyzed by either hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (CQT) or hydroxycinnamoyl:shikimate hydroxycinnamoyltransferase (CST) and microsomal 3'-hydroxylase activities.

The fate of caffeoylshikimate *in vivo* is unknown, but two considerations deserve further attention. The parsley 4-coumaroyl-CoA hydroxylase mentioned above can be active only as long as ascorbate is available in the cells. This is the case for 20 h at most following the addition of elicitor (see below). Later, the cells might rely on the shikimate ester to cover their needs for caffeic moieties. Alternatively, caffeoylshikimate might be a substrate for an additional, probably polymerizing, enzyme activity. Crosslinking and polymerization of caffeic esters might be catalyzed by peroxidases (17,19).

FORMATION OF ESCULETIN

The elicitor-induced formation of *ortho*-diphenolic acids and esters suggested that the cultured plant cells might also possess polyphenol oxidase (*o*-diphenol oxidase) activity. Parsley cells developed a soluble enzyme activity upon elicitor treatment that catalyzed the conversion of caffeic acid to esculetin (Fig. 3) (35). The reaction was completely inhibited by low concentrations of ascorbic acid or dithiocarbamate *in vitro*. Although further details of the properties of this parsley enzyme are not yet available, we assume that the enzyme represents an *o*-diphenol oxidase analogous to the corresponding chloroplast-associated enzyme described previously by Satô (59). The occurrence and relevance of plant polyphenol oxidases have been reviewed by Mayer and Harel (46). Most of these enzymes possess low substrate-specificity and changes in their activity are common upon infection of plants. The parsley polyphenol oxidase referred to here, however, showed a very narrow substrate specificity, since neither 4-coumaric acid nor caffeoyl-CoA,



R = H	trans-4-coumaric acid	R = H	umbelliferone
R = OH	trans-caffeic acid	R = OH	esculetin
R = OCH_3	trans-ferulic acid	R = OCH_3	scopoletin

Fig. 3. Conversion of *trans*-caffeic acid (R = OH) to esculetin (R = OH) by an elicitor-induced enzyme activity from cultured parsley cells.

caffeoylshikimate or chlorogenic acid could serve as substrate, the latter being a standard substrate for many other oxidases (44,46,54,67,74). Furthermore, provided no H_2O_2 was present in the assay, no product other than esculetin was found to be formed *in vitro*.

The biosynthesis of esculetin from caffeic acid and not from umbelliferone is reminiscent of the formation of scopoletin from ferulic acid (Fig. 3) as confirmed in TMV-infected tobacco (16). It is interesting to note that, in sweet potato, for example, the induction of scopoletin synthesis occurs concomitantly with that of esculetin and also of umbelliferone (67).

The enzyme activity catalyzing the formation of esculetin increased sharply approximately 25 h after addition of the elicitor to the cells (35). This increase was not directly due to a decreased level of ascorbate in the cell extracts, since the extracts were routinely desalted prior to the assays. Crude extracts prepared from the cells shortly after addition of the elicitor, on the other hand, must have contained sufficient ascorbate to sustain the hydroxylation of 4-coumaric acid and its CoA-ester. Provided that the hydroxylating activity and the polyphenol oxidase activity are not compartmentalized *in vivo*, a fine tuning must exist between the reduction in ascorbate level and the induction of the polyphenol oxidase activity, since otherwise this enzyme would oxidize ascorbate in a futile cycle. A close correlation between ascorbate level and polyphenol contents was demonstrated previously in various plants (36). It is this tuning that was suggested as being responsible for resistance expression of different *Ribes nigrum* L. genotypes against powdery mildew infection, where growth of the fungus in susceptible shoots was not suppressed "because the oxidation of phenolics is restrained by the high quantity of ascorbic acid" (66). It appears more likely, however, that the efficiency of the initial hydroxylase reaction rather than the preformed level of ascorbate in the cells might offer a clue to the mechanism of disease resistance. In this sense, the quality and quantity of the plant's responses must be intimately linked to the speed of synthesis of the individual *ortho*-diphenols.

As is the case for caffeoylshikimate, esculetin could not be recovered from elicitor-induced parsley cells. Since at present no alternative is evident, we suggest that esculetin must be incorporated into polyphenolic materials, conceivably the "melanins" that discolor the induced cells and also the infection sites in parsley plants. The time courses of esculetin as well as of caffeoylshikimate synthesis compare favorably with that of melanization. This point is presently under investigation.

REGULATION OF CELLULAR pH

It will be obvious by now that the entire pattern of reactions outlined above depends on the initial hydroxylation of coumaric acid and its CoA-ester. The critical enzyme is catalytically inactive at the pH usually prevailing in the cytoplasm of plant cells (approx. pH 7.4).

There is increasing evidence that fungal cell wall components modulate the activity of plant cell membrane translocators. Strasser *et al.* (62-64) showed that the uptake of inorganic phosphate by cultured parsley cells was impaired within 2 min following the addition of elicitor to the cells. Furthermore, amino acid uptake was inhibited while a slow, passive efflux of amino acids from the cells continued in the presence of elicitor. These results indirectly confirmed the inhibition of the plasmalemma H^+-ATPase activity which is involved in amino acid transport. More recently, Low and Heinstein (42) concluded from their studies using various fluorescence dyes with different cell cultures that asymmetric fluxes into and out of the plant cell are involved in the initial events of elicitor signal transduction. A similar conclusion was reached by Pelissier *et al.* (52) on elicitor-treated roots of muskmelon, tobacco and corn.

^{31}P-NMR spectroscopy of intact parsley cells revealed additional intracellular changes occurring in response to elicitor treatment (64). As well as a redistribution of inorganic phosphate between the cytoplasm and the vacuole, a drop in the intracellular pH was registered which was accompanied by an increase in the pH of the cell culture filtrate. The vacuolar pH decreased by approximately 0.5 pH unit. The cytoplasmic pH, on the other hand, is more difficult to determine for technical reasons. In particular, the cells must be aerated during spectroscopy to keep the cytoplasmic pH as close as possible to the *in vivo* situation (43). Re-examination of aerated control cells revealed a drop in cytoplasmic pH of approximately 0.3 upon elicitor treatment. This effect became significant as early as 10 min after addition of the elicitor to the cells and reached its maximum about 1 h later. The effect was fully reversed within the next 2 to 3 h. Similar data were reported recently on cultured *Phaseolus* cells (50). In summary, the spectroscopic results clearly imply that elicitor treatment induces a shift in the cytoplasmic pH of parsley cells that occurs rapidly and to an extent which must favor the formation of caffeic acid and its CoA-ester by the hydroxylase enzyme. It is interesting to note in this context that a pH switch has also been postulated in the stress regulation in yeast (53) and *Acer* cells (45). Preliminary experiments suggest that the induction of these very general defense reactions by a pH-shift is a widespread phenomenon in plant cells.

REGULATION OF THE ACCUMULATION OF PHENOLICS

It is still premature to reconcile the results into an appropriate setting and to suggest a final regulatory model. Nevertheless, some essential features of phenolic metabolism in elicitor-treated plant cells have emerged which appear to have a relevance for plants shortly after fungal attack. Most results to date show that the responses of elicitor-treated cultured plant cells can be compared with those of infected plants (28,47,60).

One of the prime responses of many plants to fungal attack or wounding is the formation of callose. Kauss (33) reported recently that callose synthesis *in vivo* becomes activated within a few minutes following an inducing stimulus. This activation is due to an increase in calcium concentration. It was suggested that this additional calcium is derived from the extracellular space. The time course of callose induction with concomitant induction of alkali-soluble phenolic compounds closely followed the time course described previously for the induction of persistent effects in parsley cells (63). Calcium has also been postulated as mediating the induction of phytoalexin accumulation in soybean (J. Ebel, Freiburg, personal communication) and carrot cells (39). The rise in calcium concentration must precede the formation of, for example, papillae since these, at least in their core, consist of callose. The papillae also bind calcium, as can be demonstrated with calcium-binding dyes (K. Mendgen, Konstanz, personal communication).

Rapid 'lignification' of callose was observed in wheat to be initiated as soon as papillae were visible (56). Provided that cinnamoyltransferases are active in the plant cells, however, callose or other cell constituents may become esterified rapidly with 4-coumaric acid, since the activities of phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and 4-coumarate:CoA ligase are usually sufficient *in vivo*. Several recent reports described the accumulation of cinnamic esters rather than lignin at infection sites in plants and also in elicitor-treated cultured cells (7,14,22,33). Aist *et al.*,* for example, demonstrated that ml-o resistance of barley to powdery mildew is closely correlated with the coumaric and ferulic acid content of papillae, although it should be noted that caffeic acid might have been degraded under the conditions of hydrolysis. Interestingly, the papillae which had been classified previously as "lignified" did not fluoresce any longer in the presence of the callose-specific dye "sirofluor", and neither their autofluorescence (51) nor the incorporation of cinnamic acid precursors into the papillae distinguishes the ester-linked cinnamic acids from lignins. Finally, since the induction of cinnamyl alcohol dehydrogenase activity also does not prove lignification (22) and cinnamic CoA-ester oxidoreductase activity was not induced in elicitor-treated plant cells, it is more probable that the first line of the plant's resistance responses is based on cinnamic esters and their derivatives.

Elicitor treatment induced a decrease in pH of plant cells within a period of approximately 60 min. This pH drop would be capable of activating the 4-coumaroyl-CoA-specific hydroxylase activity. The caffeoyl-CoA-specific O-methyltransferase, supplying feruloyl-CoA, showed considerable activity in non-treated control cells but nevertheless reached its maximal activity almost concomitantly with phenylalanine ammonia-lyase. All the reactions mentioned above proceed basically without requiring *de novo* enzyme induction and therefore may be critical for the rapid response in the

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plant-fungus encounter. Enzyme induction, on the other hand, usually requires at least 10 hours for maximal activities. The CoA-ester-specific hydroxylation and methylation therefore seem to be part of the first line of defense. A time-dependent change in the quality of the cinnamic esters produced in the plant might even explain the concentric structuring of papillae frequently observed by electron microscopy.

Esculetin and caffeoylshikimate were produced in elicitor-treated parsley cells considerably later than the various cinnamic CoA-esters and are subject to further metabolism. The time course of their synthesis suggests that they are required at a time when phytoalexins are also being accumulated. We assume that these compounds are incorporated *in vivo* into polymers, probably by the action of peroxidases, and that these polymers permanently cover and protect the living cells surrounding the infection site, particularly from the high concentration of phytoalexins in the infection site.

It should be noted that not all cells, both in a plant tissue and most likely also in a cell culture, show identical reactions. This is easily demonstrated in, for example, soybean root infected by *Phytophthora megasperma*, where callose accumulation does not occur in all cells (A. Bonhoff, Freiburg, personal communication). We are not yet in a position to explain this phenomenon, but it indicates that either the enzymatic capacity between individual cells differs or that the signal transduction kinetics in the resistance response may be different from one cell to the other. The latter possibility may form the basis of the induced systemic resistance.

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