Genes Expressed in *Ascochyta rabiei*-Inoculated Chickpea Plants and Elicited Cell Cultures as Detected by Differential cDNA-Hybridization

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In response to the exogenous application of elicitors and attempted invasion by pathogens, plants exhibit a wide range of defense reactions. To understand the defense mechanisms at the level of gene activation and deactivation, differential screenings were performed to isolate cDNA clones which are differentially expressed in pathogen-inoculated resistant chickpea plants and elicitor-treated cell cultures. A plenty of genes were isolated and arranged in 5 groups, namely defense-related pathways, signal transduction pathways, regulation of gene expression, catabolic pathways and primary metabolism. Most of these genes were activated although several genes were also found to be suppressed. We discuss the plausible functions of cDNA products in plant defense responses. The cDNAs provide a variety of tools to investigate molecular mechanisms of defense responses and clearly reflect the massive genomic and metabolic changes which occur during manifestation of antimicrobial defense.

Introduction

Plants exhibit a wide range of defense responses against microbial phytopathogens (Lamb *et al.,* 1989; Barz *et al.,* 1990; Kombrink and Somssich, 1995), which can be classified into two groups; 1) the first group of responses is very rapid, because it occurs within seconds to minutes after perception of the microbial signals. These responses do not require *de novo* gene expression such as the generation of reactive oxygen species. 2) the second expressed group of responses is rather late, because it is observed within minutes to hours or days after signal perception. It should require *de novo* gene expression and protein synthesis. The later defense responses are so called active defenses, including the reinforcement of cell walls, the production of pathogenesis-related (PR) proteins and the accumulation of phytoalexins. The active defense response-related genes and their proteins are relatively well investigated. In the interaction between chickpea (*Cicer arietinum* L.), an important leguminous crop plant, and its potent fungal pathogen, *Ascochyta rabiei*, phytoalexin biosynthetic enzymes and PR-proteins had intensively been investigated (Barz *et al.,* 1990; Daniel *et al.,* 1990a, 1990b; Vogelsang *et al.,* 1994; Vogelsang and Barz, 1990, 1993a).

In a series of our investigations, in addition to whole plants, we have established and utilized chickpea cell suspension cultures from both *A. rabiei* resistant and susceptible cultivars (Kessmann and Barz, 1987). Since the characteristics of both cell culture lines well corresponded to those observed for the *in planta* system, cell suspension cultures provide a suitable model system for studying gene expression and metabolism in defense responses (Weigand *et al.,* 1986; Kessmann *et al.,* 1988; Daniel *et al.,* 1990a). Through protein purification and amino acid sequencing, we have cloned cDNAs encoding NADPH: isoflavone oxidoreductase (Tiemann *et al.,* 1991) and class III acidic chitinase (Vogelsang and Barz, 1993b). However, in addition to those genes, there should be other genes contributing to the establishment of defense responses. Therefore, to comprehensively investi-

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gate the defense responses, we have attempted to isolate cDNAs which show differential expression in the *A. rabiei*-inoculated resistant chickpea cultivar compared to non-inoculated control plants, and in elicited and non-elicited cell cultures by differential hybridization techniques.

In the present report, we describe the structural analyses of the isolated cDNAs and their plausible functions in the defense response by homology analyses. The cDNAs obtained were classified into five groups as 1) defense related pathways, 2) signal transduction pathways, 3) regulation of gene expression, 4) catabolic pathways and 5) primary metabolism. We also discuss the roles of the relevant enzymes in the plant-microbe interaction.

We present in detail the induced and suppressed expressions of four genes of the isolated cDNAs as representative examples. Furthermore, we discuss the differences between cell cultures and organized whole plants in the defense response against microbial pathogens.

Materials and Methods

Plant materials and fungal inoculation

The chickpea (*Cicer arietinum* L.) ILC3279 (resistant) and ILC1929 (susceptible) plants were grown as described by Höhl *et al.* (1990), and 2 weeks old plants were used for inoculation experiments. A chickpea pathogen, *A. rabiei* isolate 21, was sporulated on agar plates containing autoclaved chickpea seeds. The harvested pycnidiospores were adjusted to a concentration to 1×10^6 spores/ml in water. The aerial parts of the chickpea plants in an incubation container were inoculated with 10 ml of *A. rabiei* spore suspension by spraying. The mature leaves in the middle part of the plants were harvested at the indicated time points, and were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Cell cultures and elicitation

Cell-suspension cultures of *C. arietinum* L. (resistant cultivar ILC 3279) were grown in a modified PRL 4c-medium as described by Kessmann and Barz (1987). For the elicitation of the cells, yeast extract (Difco Laboratories, Detroit, Mich., USA) was added to the culture medium at 5 mg/ml of final concentration 3 days after subculturing. Cells were harvested by filtration, washed with water and immediately used for extraction. For northern blot hybridization experiments cultured cells were challenged with a Phytophthora megasperma elicitor preparation (Ayers *et al.,* 1976).

RNA extraction

Total RNA was isolated from frozen leaves or cultured cells with the methods described by Sambrock *et al.,* (1989) for the construction of cDNA libraries and by Chomczynski and Sacchi (1987) for Northern blot analysis. Poly(A)$^+$-RNA was isolated from total RNA with polyAT tract mRNA Isolation System (Promega, Mannheim, Germany) according to the supplier's instructions.

cDNA library construction and differential screening

cDNA libraries were constructed from both plant and cell culture of *C. arietinum* L. cv ILC3279, resistant cultivar to *A. rabiei*. Poly(A)$^+$-RNAs were purified from chickpea leaves harvested 4 days after inoculation of *A. rabiei*, and from cell cultures harvested 6 hr after application of yeast extract elicitor. Then, cDNA libraries were constructed with ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Differential screening was performed as described by Sambrock *et al.,* (1989). Thirty-five thousand independent cDNA clones from both libraries were subjected to the differential hybridization. ^{32}P-cDNA probes were generated from 5 µg of poly(A)$^+$-RNA of control cell culture (water, 6 hr-treatment), elicited cell culture (elicitor, 6 hr-treatment), control healthy chickpea plants and plants 4 days after inoculation with *A. rabiei* with reverse transcriptase (New England Biolabs, Schwalbach/Taunus, Germany). Heat-linearized poly(A)$^+$-RNA (5 µg in 5 µl of water) at 70 °C for 1 min, was mixed with 50 U (1.5 µl) of RNase inhibitor (RNAguard, Pharmacia), 3 µl of 10 × RTase buffer (NEB), 5 µl (2.5 mg/ml) of random primer (Pharmacia), 1 µl of labeling mix-ATP (dCTP, dGTP, dTTP; 20 mM, Pharmacia), 1 µl of DNA polymerization mix (120 µM dNTPs, Pharmacia), 10.5 µl of a-^{32}P-dATP (105 µCi, NEN) and 3 µl of M-MuLV reverse transcriptase (75 U, NEB), then incubated at 37 °C for 1 h. Reaction was terminated by the addition of 2.4 µl of 0.25 M EDTA (pH 8.0) and 1.2 µl of 10%

SDS. After addition of 3.7 µl of 3 N NaOH, the reaction was incubated at 68 °C for 30 min to hydrolyze template RNA. To neutralize 12.4 µl of 1 M Tris-HCl (pH 7.5) and 3.7 µl of 2 N HCl were added, then mixed with 50 µl of TE buffer (pH 8.0) prior to phenol/chloroform extraction and ethanol precipitation. Each ^{32}P-labelled cDNA probe was counted to adjust the specific radioactivity and then hybridized with a duplicated nylon membrane, Qiabrane (Qiagen, Hilden, Germany) made from cDNA plaques. The hybridizations were performed in 5 × Denhart's solution containing 4 mM EDTA, 0.5% SDS, 25 mM KH_2PO_4 and 6 × SSC at 62 °C overnight and then finally washed in 0.5 × SSC and 0.1% SDS at 62 °C. cDNA clones which seemed to be differentially expressed, were isolated after autoradiography, and pBluescript-phagemids containing cDNA inserts were formed by in vivo excision procedure as described in the manufacture's protocol.

Sequencing analysis

The DNA sequences of cDNA clones were determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

Northern blot analysis

Hybridization was performed as described by Sambrock *et al.* (1989). Ten microgram of total RNA was denatured, fractionated by electrophoresis on a 1% agarose gel and then blotted onto a nylon membrane filter with a downward alkaline capillary transfer method (Chomczynski, 1992). DNA- and RNA-hybridization probes were made with ^{32}P-labeled dATP or digoxigenin (DIG)-UTP. In the case of radioisotope labeling, inserted DNA fragments of cDNA clones were excised by digestions with appropriate restriction enzymes, and ^{32}P-dATP was incorporated by random priming method. Hybridization was performed at 42 °C overnight in a solution containing 50% (v/v) formamide, 4 × SSC, 5 × Denhardt's, 0.2% SDS (w/v) and 0.12 mg/ml of heat denatured salmon sperm DNA. Final washes were at 55 °C in a solution containing 0.1 × SSC and 0.1% SDS. On the other hand, DIG-labeled RNAs were made using RNA transcription kit (Stratagene) with DIG RNA labeling Mix (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed at 68 °C overnight in a solution containing 50% (v/v) formamide, 5 × SSC, 0.1% laurylsarcosine, 0.02% SDS (w/v) and 2% Blocking solution (Boehringer Mannheim). Final washes were at 68 °C in a solution containing 0.1 × SSC and 0.1% SDS. Hybridized mRNAs were detected with anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim) and its chemiluminescent substrate, CDP-star.

Results and Discussion

Isolation of cDNAs

The size of cDNA libraries constructed from cultured cells 6 h after elicitation and chickpea leaves 4 days after fungal inoculation was 1.75×10^6 pfu/mg poly(A)$^+$-RNA and 2.35×10^6 pfu/mg poly(A)$^+$-RNA, respectively. After one round of differential hybridization, 109 and 61 independent cDNA clones were isolated from cDNA libraries of inoculated chickpea plants and elicited cell cultures as candidates for differentially expressed genes. Clones were designated as INR (inoculation responsive) and ELR (elicitor-responsive) clones, respectively. The nucleotide sequences of about 75% of the isolated cDNA clones were completely or partially determined, and compared with previously reported DNA databases and/or protein databases by the BLAST search protocol. For some 70% of the sequenced cDNAs significant degrees of homology could be found. In Table I, cDNA clones which show significant sequence homology to reported genes and/or proteins are listed. cDNAs were classified in 5 areas of plausible functions of the gene products as described below.

cDNAs involved in defense related pathways

In the cDNA library from inoculated chickpea plants, a number of defense related genes were found. One major group are genes coding for glycine rich proteins (GRP, INR23 and 16 other clones). They were later classified into two subgroups by their structural analyses, and both are strongly induced in plants after inoculation (Cornels, Ichinose and Barz, unpublished). In Fig. 1, we show an expression profile of GRP genes after inoculation in plants using INR23 as a probe for northern blot analysis. GRPs express a representa-

Table I. Isolated cDNAs and their homologies and plausible functions.

Plausible function of the gene product[a]	Homology[a] (organism[a], a. n.[c])	Representative cDNA[d] (a. n.[c])
1. Defense related pathways		
Reinforcement of cell walls	GRP: glycine rich protein (alfalfa, pir S18345)	INR5 (AJ007556)
PR-proteins	acidic β-1,3-glucanase (tobacco, pir. JQ0982)	INR9 (AJ012751)
	basic β-1,3-glucanase (pea, pir S28430)	INR15 (AJ131047)
	chitinase (pea, pir S56694)	ELR2 (AJ012821)
	osmotin-/thaumatin-like protein (tobacco, pir JH0230)	ELR112 (AJ010501)
Phytoalexin biosynthetic enzymes	chalcone isomerase (alfalfa, pir S44371)	ELR17* (AB024988)
	chalcone reductase (alfalfa, pir S48849)	ELR24 (AB024989)
	isoflavone reductase (chickpea, pir S17830)	ELR30* (AB024990)
	cytochrome P450 hydroxylase (eggplant, pir S38535) (flavonoid 3′-3,5′-hydroxylase)	INR142* (AB026263)
ROS scavenging enzymes	catalase (soybean, pir S20999)	INR37 (AJ131046)
	NaDPH oxidoreductase (*A. thaliana*, pir S57614)	INR34 (AB027757)
	ascorbate peroxidase (pea, pir A45116)	ELR20 (AB024991)
Multidrug resistance protein[e]	transporter (rat, pir 50217)	INR116 (AB024992)
Heat shock proteins[e]	chaperonine (tobacco, pir JQ1360)	ELR36* (AB024993)
2. Signal transduction		
Protein (de)phosphorylation	protein kinase (alfalfa, pir S37643)	INR28 (AJ131048)
	protein phosphatase (pea, pir S40171)	INR45 (AJ131045)
Signal transducer	*rab/ypt* type small GTP binding protein (maize, pir B38202)	INR134 (AB024994)
	rab/ypt type small GTP binding protein (pea, pir S33531)	ELR19 (AB024995)
	rac/rho type small GTP binding protein (pea, pir A47525)	ELR26 (AB024996)
Function unknown	HR inducible gene[f]	INR129 (AJ131049)
3. Gene expression		
Transcription factors	bZIP DNA binding protein (tomato, pir S52203)	INR127* (AB024997)
	Zn (Ring) finger protein[g] (soybean, pir S49445)	INR132* (AB026262)
Translation	translation elongation factor (maize, pir S66338)	INR39* (AB024998)
4. Catabolic pathways		
Proteolysis related proteins (amino acids recycling)	cystein proteinase (pea, pir S11862)	INR8 (AJ009878)
	aspartate proteinase (tomato, pir S715591)	INR21* (AB024999)
	multicatalytic endopeptidase complex (*A. thaliana*, pir S29240)	INR54 (AB025000)
	ubiquitin (parsley, pir S30151)	ELR29* (AB025001)
5. Primary metabolism		
Carbohydrate metabolism	ribulose-1,5-bisphosphate carboxylase (pea, pir A27874) (small subunit)	INR145 (AJ131050)
	chlorophyll *a/b* binding protein (pea, pir A26780)	INR133 (AJ131044)
	fructose-1,6-bisphosphate aldolase (pea, pir S58167)	ELR38* (AB025002)
	plastoquinol-plastocyanin reductase (pea, pir S26199)	INR122* (AB025003)
	transketolase (potato, pir, S58083)	INR121* (AB025004)
	pyruvate kinase (potato, pir, JC1481)	INR6* (AB025005)
	sucrose transport protein (*A. thaliana*, pir S38197)	INR27* (AB025006)
	malate dehydrogenase (kidney bean, pir S48198)	ELR35* (AB025007)

[a] Plausible functions which were expected based on the homology analysis of the nucleotide sequences, and which are classified into five groups and further several subgroups.
[b] Organisms were described by only one species as representative.
[c] Accession numbers (a. c.) registered in protein or DNA databases.
[d] cDNAs were designated as INR (inoculation responsive) or ELR (elicitor responsive) clones. Asterisks indicate that only the partial nucleotide sequence of cDNA clone was determined.
[e] The involvement of these gene products in the plant defense response against pathogens has not been reported so far.
[f] see text.
[g] Ring H2 finger proteins were not yet proved as transcription factors in plants (see text).

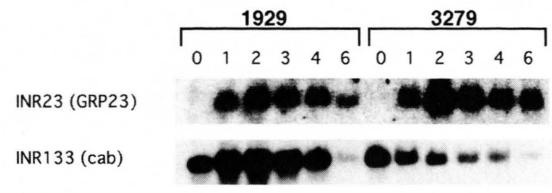

Fig. 1. Northern blot hybridization analyses to detect mRNA for GRP (INR23) and chlorophyll *a/b* binding protein (INR133) in response to the inoculation with *A. rabiei* 21 susceptible (ILC1929) and resistant (ILC3278) chickpea cultivars. Total RNAs were prepared from the leaves of control (uninoculated, indicated as 0) and 1, 2, 3, 4 and 6 days post inoculation (dpi) as indicated.

tive pattern of mRNA accumulation for the inoculation-induced genes in plants. Although there were no signals in uninoculated control plants, 1.0 kb of transcript was rapidly and strongly induced within 1 day after inoculation in both ILC1929 and 3279 chickpea cultivars. The hybridized signals in ILC3279 are somewhat stronger than in ILC1929. GRPs are thought to contribute to the reinforcement of cell walls and xylem elements against pathogen attack. There are further notable points; that is 1) all cDNA clones related to strengthening of cell walls were found in the cDNA library of the inoculated plants, and 2) although GRP is constitutively expressed in chickpea cell cultures, the application of elicitor resulted in significant suppression of its gene expression (Cornels, Ichinose and Barz, unpublished). The detected suppression of GRP expression in cell cultures indicates that the responses of cells in suspension culture to elicitor treatment may be quite different from responses found in plants. The reason might be due to the characteristics of "suspended cultures cells" which fail to form organs or tissues and possess no organized structures like plants. The molecular mechanism of the suppression of GRP genes in elicited cell cultures remains to be elucidated.

Among the cDNA clones isolated from elicited cell cultures, there are three cDNAs encoding the enzymes for pterocarpan phytoalexin biosynthesis. The sequence of ELR30 was identical to the previously isolated isoflavone reductase (IFR)-cDNA in chickpea (Tiemann *et al.,* 1991), and sequences of ELR17 and ELR24 show high homology with the previously isolated chalcone isomerase (CHI) in alfalfa (McKhann and Hirsch, 1994) and chalcone reductase (CHR) in soybean (Welle *et al.,* 1991), respectively. In addition to the above clones, we found that INR142 has a homologous sequence to cytochrome P-450 hydroxylase such as flavonoid 3′,5′-hydroxylase from eggplant (Toguri *et al.,* 1993). Although we have not yet defined the function of INR142, this gene might be involved in the phytoalexin biosynthetic pathway (Barz and Welle, 1992). In Fig. 2, a time course study of the IFR-mRNA in the elicitor-treated ILC3279 cell culture is depicted, as a typical pattern for the expression of elicitor-induced cDNA clones. Although there is little IFR-mRNA in untreated and water-treated cells, the mRNA started to accumulate within 2 hr and reached maxima 4 hr after application of the elicitor. In the cell suspension culture of the resistant chickpea cultivar ILC3279 phytoalexins start to accumulate within 4 hr and reached maxima 12 hr after elicitor application (Daniel *et al.,* 1990a). Thus, the isolation of the above cDNA clones from elicitor-treated cell cultures very well coincide with the previous observation.

The sequence analyses revealed that INR9 and INR15 encode acidic and basic β-1,3-glucanase, respectively, and showed 59% homology to each other at the nucleotide sequence level (Hanselle, Ichinose, Tanaka and Barz, unpublished). Clone ELR2 showed high homology to class I basic endo-chitinase. INR9 and ELR2 seem to contain the full length of an open reading frame in each cDNA clone, and the molecular weights of the putative proteins are deduced as 35.6 kDa for INR9

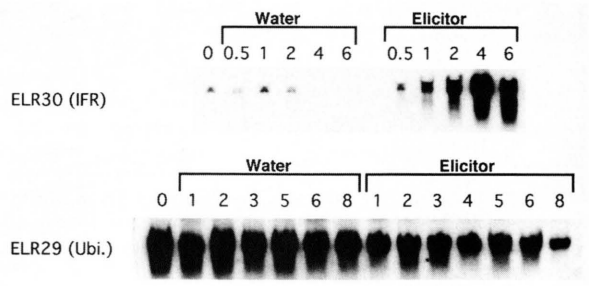

Fig. 2. Northern blot analyses for IFR-mRNA (ELR30) and ubiquitin (ELR29) in chickpea ILC3279 cell culture. Total RNAs are prepared from the control cells (untreated, indicated as 0), water-treated cells and *Phytophthora megasperma* elicitor-treated cells at indicated time points (hours).

and 35.5 kDa for ELR2. Since ELR2 also seems to contain signal peptides, the molecular weight for ELR2 as a mature protein was calculated to be 32.8 kDa. The protein which shows the highest homology to INR15 (putative molecular weight of the mature protein is 35 kDa) is a β-1,3-glucanase from pea, and its putative molecular weight was 34.9 kDa as the mature form (Chang *et al.,* 1992). We have previously purified a 36 kDa basic β-1,3-glucanase and a 32 kDa basic endo-chitinase from chickpea cell cultures (Vogelsang and Barz, 1993a). The molecular weights of the purified proteins of basic β-1,3-glucanase and basic chitinase were well coincide with the putative molecular weights of the products of INR15 and ELR2, respectively. Thus, the corresponding protein products of INR15 and ELR2 might be identical to the purified 36 kDa basic β-1,3-glucanase and 32 kDa basic chitinase, respectively.

Furthermore, ELR112 was identified as a cDNA clone for an osmotin/thaumatin-like protein (TLP). In addition to the sequence analyses of ELR112, we have purified from chickpea another TLP designated as PR-5a, and isolated its cDNA clone. Biochemical investigation on these TLPs in chickpea will be published elsewhere (Hanselle and Barz).

cDNA clones encoding proteins homologous to enzymes which are able to scavenge reactive oxygen species (ROS) were also obtained from the libraries. Thus, INR37 and ELR20 showed very high homology to many plant catalases and ascorbate peroxidases, respectively (Isin and Allen, 1991; Mittler and Zilinskas, 1991). Furthermore, INR34 showed significant sequence homology to *Arabidopsis thaliana* P4 cDNA which provides tolerance to yeasts towards the thiol-oxidizing drug, diamide (Babiychuk *et al.,* 1995). Since P4 cDNA has a homology to the ζ-crystallin-like proteins in the C-terminus, and lens crystallin has been shown to possess NADPH: quinone oxidoreductase activity (Rao *et al.,* 1992), the product of P4 cDNA is thought to be a NADPH oxidoreductase (Babiychuk *et al.,* 1995). As a very early event of recognition of a pathogen in plants, it is well known that ROS are generated, such as superoxide anion and hydrogen peroxide (Bolwell and Wojtaszek, 1997; Otte and Barz, 1996). However, since we detected such cDNA clones in this study, the genes encoding ROS scavenging enzymes might still be activated to detoxify products generating oxidative stress at the relatively later stage of the defense response, such as 4 days after fungal inoculation of plants. In tobacco plants, induction of the mRNAs encoding cytosolic superoxide dismutase and ascorbate peroxidase were observed during the hypersensitive response caused by tobacco mosaic virus (Mittler *et al.,* 1996, 1998). We have also observed the induced expression of the mRNAs encoding catalase (INR37) in elicitor-treated cell cultures (data not shown).

The isolated cDNA clone INR116 showed significant sequence homology to a multidrug resistant protein (MDR) homolog in *A. thaliana* (Dudler and Hertig, 1992). Although, the function and expression pattern of plant MDR genes are not clear yet, it is plausible that MDR might be induced after inoculation to transport toxins produced by pathogens to the outside of the cells. In this context it should be mentioned that *A. rabiei* produces a series of phytotoxic solanapyrones which might be subject to MDR-mediated transport (Benning and Barz, 1995).

Some of the genes encoding PR-proteins and phytoalexin biosynthetic enzymes are induced not only by pathogen attack or elicitor-treatment but also other environmental stimuli, such as wounding, osmotic stress and exogenous application of plant hormones. Heat shock is one of such major environmental stress factors, and the proteins induced by heat treatment might be involved in the general plant defense mechanisms. The sequence of ELR36 is homologous to a member of the heat shock protein (HSP) 70 family (Denecke *et al.,* 1991). A report on HSP gene expression in plant defense against phytopathogens describes HSP90 gene as being induced in *Erysiphe graminis*-inoculated barley (Walther-Larsen *et al.,* 1993). Furthermore, *prp1*, a gene encoding a pathogenesis-related protein in potato showed striking similarity to that of a 26-kDa HSP in soybean (Czarnecka *et al.,* 1988; Taylor *et al.,* 1989). However, the contribution of HSP gene expression in plant defense against phytopathogens is not yet clear.

cDNAs (possibly) involved in signal transduction

Numerous reports postulate an involvement of protein phosphorylation and dephosphorylation in plant defense responses including the discovery of

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the Ser/Thr type protein kinases as products of plant resistant genes, such as *Pto* in tomato (Martin *et al.,* 1993) and *Xa21* in rice (Song *et al.,* 1995). In this connection, it is very interesting that we found cDNA clones encoding protein kinase (INR28) and protein phosphatase (INR45). INR28 showed the highest homology to an alfalfa MsK family of protein kinases which belong to the shaggy/glycogen synthase kinase-3 (Pay *et al.,* 1993). Shaggy protein kinase is known to regulate the activity of the transcription factor involved in the developmental regulation of *Drosophila melanogaster* (Bourois *et al.,* 1990). Glycogen synthase kinase-3 (GSK-3) is known to phosphorylate not only glycogen synthase but also transcription factors c-jun and c-myb in rat (Plyte *et al.,* 1992). Thus, the phosphorylation of c-jun by GSK-3 blocks its DNA binding activity (Plyte *et al.,* 1992). It has been reported that a fungal elicitor leads to rapid phosphorylation of some specific proteins in parsley cell suspension cultures (Dietrich *et al.,* 1990). Furthermore, exogenous application of protein kinase inhibitors, K252a and/or staurosporine, inhibit the expression of plant defense responses such as elicitor-induced oxidative burst and insolubilization of cell wall proteins in chickpea (Otte and Barz, 1996), accumulation of phytoalexin in pea (Shiraishi *et al.,* 1990) and induction of basic PR-proteins in tobacco (Raz and Fluhr, 1993). A transcription factor, H/GBP-1 which binds to elicitor-responsive *cis*-elements, H- and G-box in bean *CHS15* gene promoter was found to be transported into nuclei after phosphorylation by Ser kinase (Droge-Laser *et al.,* 1997). On the other hand, sequence analysis of INR45 revealed high homology to pME1, a cDNA clone for the regulatory subunit of type 2A Ser/Thr phosphatase in pea (Evans *et al.,* 1994), and its homologs in other organisms. The application of protein phosphatase 2A inhibitor, such as calyculin A or cantharidin, resulted in medium alkalinization and strong activation of 1-aminocyclopropane-carboxylate synthase in tomato suspension cell culture (Felix *et al.,* 1994) and the induction of oxidative burst in chickpea cell suspension culture (Otte and Barz, 1996). Protein phosphorylation is also reported to be positively involved in ethylene- and wound-induced basic PR gene expression as mentioned above (Raz and Fluhr, 1993). On the other hand, protein dephosphorylation is reported to be oppositely involved in the salicylic acid-induced acidic PR gene expression (Conrath, *et al.,* 1997). Studies on the expression pattern together with the analysis of the target proteins of INR45 in chickpea are needed.

Three distinct cDNAs (INR134, ELR19 and ELR26) were isolated encoding *ras*-related small GTP-binding protein homologs. Small GTP binding proteins have been implicated in the regulation of growth, differentiation, cytoskeletal organization and intracellular transportation of cells, and they have been reported from a variety of organisms, including mammals, insects, yeast and higher plants (Hall, 1994; Terryn *et al.,* 1993). Small GTP-binding proteins constitute a multigene-family, and based on structural analyses they have been grouped in three major subfamilies, *ras, rho/rac* and *rab/ypt*, and some minor subfamilies (Hall, 1994; Terryn *et al.,* 1993). Transgenic tobacco plants expressing *rgp1*, a gene encoding a *ras (ypt)*-related GTP-binding protein isolated from rice (Sano and Youssefian, 1991) resulted in abnormal induction of salicylic acid and expression of genes for basic PR-protein in response to wounding (Sano *et al.,* 1994; Sano and Ohashi, 1995). INR134 and ELR19 were classified into the *rab/ypt* subfamily, whereas ELR26 was placed into *rho/rac* subfamily by the sequence analyses. Investigations on the expression and function of these genes in chickpea are now underway.

INR129 showed high homology to patent DNA (gb: I38527, I57002, I59868) and tobacco cDNA NG1 (gb: NTU66271, Karrer *et al.,* 1998). Patent DNA is registered in the DNA database as "chemically regulatable and anti-pathogenic DNA sequence", however details of this DNA including the source are not published yet. Tobacco NG1 has been reported with a hypothetical open reading frame, which is claimed to induce HR-like lesions. These references suggest that INR129 might also be involved in hypersensitive response or some defense responses. INR129 also has a low but significant homology with mammal band 7 membrane protein (Hiebl-Dirschmied *et al.,* 1991) and its homologs in procaryotes (Nobel *et al.,* 1993). Human band 7 protein is thought to be involved in the regulation of an ion-channel (Stewart *et al.,* 1993). Taken together, INR129 might be involved in plant defense response as well as other homologous DNA sequences, although the func-

tion of INR129 product is not clear at all, including its correlation with the signal transduction pathway.

cDNAs (putatively) involved in gene expression

Several chickpea cDNA clones were grouped together because of putative gene product function in basic steps of gene regulation. bZIP proteins are well known as transcription factors in plants, and are involved in the regulation of a wide variety of plant genes. Clone INR127 was found to belong to such bZIP proteins because it showed high homology to vsf-1 proteins in tomato; these proteins were recognized to bind to *cis*-regulatory elements for vascular-specific gene expression (Torres-Schumann *et al.,* 1996).

The hypothetical protein of INR132 possesses a plausible zinc finger domain constituting a Cys_3–His_2–Cys_3 motif. As transcription factor, many zinc finger proteins are documented from mammals, insects, yeast and higher plants. However, among the Zn finger protein families, the function in plants of ring H2 finger proteins possessing Cys_3–His_2–Cys_3 motif is not yet elucidated, including DNA binding activity. There are sequence reports on the isolation of cDNA clones possibly encoding ring H2 finger proteins (putative zinc finger proteins: PZF) from *Lotus japonicus* and soybean (Schauser *et al.,* 1995) as well as from elicitor-treated pea epicotyls (Ichinose *et al.,* 1999). Since ring finger proteins possessing a Cys_3–His_1–Cys_4 motif are clearly characterized as DNA binding transcription factors in plants, such as *COP1* in *A. thaliana* (Deng *et al.,* 1992), the product of INR132 might also possess DNA binding activity and thus be involved in the defense responses. Its exact role clearly deserves further investigation due to its important function.

cDNAs, INR39 and INR149, were isolated which encode translation elongation factor-1 alpha chain (EF-1 α). EF-1 α represents a highly conserved protein, and it was cloned from a variety of plant species (Pokalsky *et al.,* 1989). Several reports describe that the gene products of some defense genes such as 3-hydroxy-3-methylglutaryl (HMG) CoA reductase in potato (Yoshioka *et al.,* 1996) and ascorbate peroxidase in tobacco (Mittler *et al.,* 1998) are post-transcriptionally regulated, a translational elongation event might also be involved in the regulation of defense gene expression in chickpea.

cDNAs involved in catabolic pathways

Infection-induced changes in plant cellular metabolism also comprise several catabolic pathways which may provide energy and substrates for the formation of inducible defense-related routes.

There are several cDNA clones whose gene products appear to be involved in proteolysis, such as cysteine proteinase (INR8, INR124), aspartate proteinase (INR21) and multicatalytic endopeptidase complex (INR54). Clone INR8 codes for a 362 amino acids comprising preprocysteine proteinase which shares very high homology with such enzymes from *Pisum sativum* and *Vicia faba* (Gitmans and Barz, unpublished). INR8 protein, however, is significantly different from an ethylene-inducible chickpea cysteine proteinase involved in the degradation of storage proteins during seed germination (Cervantes *et al.,* 1994).

We also isolated two ubiquitin cDNAs, INR12 and ELR29. During the elicitor-induced defense responses, the expression of the cell cycle related genes, such as histon H2A, H2B, H3 and H4, a mitotic cyclin and a p34 cdc2 protein kinase is repressed (Logemann *et al.,* 1995). Although the present authors didn't observe the repression of ubiquitin gene expression in plants, we suggest that elicitor-mediated signals might repress the cell cycle related genes. In chickpea cell suspension cultures, expression of ubiquitin genes showed a tendency to be suppressed by elicitor treatment (Fig. 2). Although the role of the repression of ubiquitin genes is not clear, the isolation of cDNAs related to proteolysis indicates that the turnover of amino acids in some classes of proteins (possibly the products of house keeping genes), might be accelerated by the elicitor and/or the inoculation with incompatible pathogens. Therefore, together with some classes of molecular chaperonine/heat shock proteins, the proteinases and ubiquitin might contribute to *de novo* synthesis of the products of a variety of defense genes.

cDNAs involved in primary metabolism

Many cDNAs involved in the photosynthetic pathway and glycometabolism were isolated. These were cDNAs encoding the small subunit of

ribulose-1,5-bisphosphate carboxylase (INR102 and other 4 clones), chlorophyll *a/b* binding proteins (INR133 and other 8 clones), fructose-1,6-bisphosphate aldolase (INR104, ELR31 and ELR38), plastoquinol-plastocyanin reductase (INR122), transketolase (INR121), pyruvate kinase (INR6) and sucrose transport protein (INR27). As a typical and representative pattern of gene expression whose transcripts were decreased in resistant cultivar ILC3279 after inoculation with *A. rabiei*, we show a Northern blot analysis of chlorophyll *a/b* binding (cab) proteins (Fig. 1). When we used INR133 as hybridization probe, a 1.0 kb signal was drastically reduced and almost disappeared 6 days post inoculation. However, in the susceptible cultivar ILC1929, the amount of transcripts was first increased within 1 to 4 days after inoculation with *A. rabiei*, and only then disappeared 6 days after inoculation. A similar suppression of genes in the resistant cultivar was also observed for other photosynthesis-related genes, such as ribulose-1,5-bisphosphate carboxylase and fructose-1,6-bisphosphate aldolase (data not shown). As described above, some products of house keeping genes might be degraded for an acceleration of turnover. Because the genes encoding cab proteins are a group of such house keeping genes in leaves, the suppression of photosynthesis-related genes might support the efficient utilization of a limited amount of substrates and apparatus for the transcription of defense genes. The mechanism for the cab gene activation in the susceptible cultivar remains to be elucidated.

ELR35 was identified as a cDNA clone encoding malate dehydrogenase. Bean malate dehydrogenase was initially erroreously isolated as a NADP-dependent cinnamyl-alcohol dehydrogenase (Walter *et al.*, 1988, 1994), however, it showed rapid induction of mRNA accumulation by elicitor-application. Malate dehydrogenase catalyzes the oxidative decarboxylation of malate to pyruvate, producing CO_2 and NADPH. This pyridine nucleotide is required for various anabolic pathways such as phytoalexin biosynthesis.

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