GENE EXPRESSION IN MESOPHYLL PROTOPLASTS

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INTRODUCTION

Higher plants are multicellular organisms, composed of tissues and organs specialised in particular functions. As a consequence, due to their position in the plant, cells present various morphologies, have different metabolisms and each cell type expresses a particular set of genes. Nevertheless, under particular circumstances, this differentiation can be lost. For example, after wounding cells reexpress their ability to grow and form a healing callus. If the damage is extensive, some cells localised near the wound reform stem or root meristems (decapitated plants, cuttings). In some plant species shoot and root meristem neoformation is not limited to wound repair, but is the basis of the natural plant propagation. Thus cell dedifferentiation is an important process in plant development. It is also the basis of *in vitro* tissue culture, since most cell lines are obtained from primary explants composed of differentiated cells. Nevertheless, the process of cell dedifferentiation in the entire plant or in large primary explants cultivated *in vitro* is difficult to analyse, due to the heterogeneity of the cell population. Protoplasts isolated from leaf mesophyll constitute a primary explant which is composed of a pippetable population of cells, all originating from the same tissue. In culture, most protoplasts follow the same development and divide.

This paper reviews our present knowledge on changes in gene expression induced in mesophyll cells by protoplast isolation, and attempts to determine if they are related to the dedifferentiation process.

MODIFICATION IN CELL STRUCTURE

Mesophyll cells present a large central vacuole and a thin cortical layer of cytoplasm which contains the nucleus and evenly distributed plastids. Protoplast isolation does not

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greatly modify this structure (Meyer and Herth, 1978). During the first two days of culture, the protoplasts become bigger and the chloroplasts surround the nucleus like a rosette, cytoplasmic strands are formed. At the same time cell wall regeneration starts and cell shape changes from round to oval. The cytoplasmic ribosome and polysome density and the number of dictyosomes increase. Cytoskeleton is profondly modified (Hahne and Hoffmann 1985; Le Guyader, 1990; Meijer *et al.*, 1988). Nuclear division takes place after two days in culture. At the same time chloroplast thylakoids are disrupted and chloroplasts change to amyloplasts. After one week in culture cells derived from mesophyll protoplasts show the morphology of callus cells (Herth and Meyer, 1978).

At the molecular level the very low thymidine incorporation observed at the begining of culture is followed after 30 hours by a massive incorporation (Zelcer and Galun, 1976,1980) and an increase of the DNA content (Galbraith *et al.*, 1981) indicating the entry into S phase. Uridine and methionine incorporation rise during the first hours in culture indicating active RNA (Coutts *et al.*, 1975; Sakai and Takebe, 1970) and protein synthesis (Bergounioux *et al.*, 1988a; Gigot *et al.*, 1975; Cooke and Meyer, 1981; Ruzicska *et al.*, 1979;).

Culture medium for mesophyll protoplasts is similar to a callus culture medium, with the addition of an osmoticum in order to replace the wall pressure (Nagata and Takebe, 1970). In the absence of hormones mesophyll protoplasts do not divide: A kinetic analysis shows that auxin is necessary from the begining of culture for the progression into the cell cycle, while cytokinin intervenes later in culture, just before S phase. In addition to this effect on mitosis, auxin and cytokinin profoundly affect the development of protoplasts in culture: In the absence of one of these hormones cells become bigger, cytoplasmic strands are rare, cell wall reformation is limited and the culture medium become toxic (Bergounioux *et al.*, 1988b; Meyer and Cooke, 1979).

Changes in a few enzymatic activities during protoplast culture have been reported: RNAse activities rise 10 to 15 times, apparently as a consequence of the osmotic pressure (Lazar *et al.*, 1973). Peroxidase activities increase in culture (Mäder *et al.*, 1977; Siminis *et al.*, 1993).

The first published electrophoretic patterns (Fleck *et al.*, 1979, 1980) show that the most abundant leaf proteins are no longer synthesized in protoplasts a few hours after isolation, and that most of the proteins synthesized by protoplasts are undetectable in the leaf. Most of these changes are independent of the composition of the culture medium (Meyer *et al.*, 1984a,b). Thus, at that time it appeared clear that changes in protein synthesis take place early during or after protoplast isolation, and that the isolation has a far more important impact than the composition of the culture medium.

CHANGES IN GENE EXPRESSION

In recent years molecular cloning of plant genes has developed rapidly and the approach to the analysis of early development has shifted from protein synthesis to mRNA analysis.

Photosynthesis Related Genes

Most of the metabolic activity in mesophyll cells is centered on photosynthesis. Both nuclear and chloroplast genes participate in the synthesis of the chloroplast. In particular ribulose 1,5-bisphosphate carboxylase (RuBP) is composed of 8 identical small subunits (SSU) encoded by the nuclear genome, associated with 8 identical large subunits (LSU) encoded by the chloroplast genome. This enzyme, located in the chloroplast stroma, catalyses the first reaction in the Calvin cycle, namely CO_2 fixation on ribulose 1,5-bisphosphate. In addition to RuBP mRNAs, the fate of mRNAs coding for a light harversting chlorophyll a/b binding protein from photosystem II (LHCII) as well as two chloroplast genes encoding a quinone fixing protein (DI) and a protein of photosystem I (P700) have been analysed during protoplastization and in culture. All mRNAs corresponding to these genes are abundant in tobacco leaf. The mRNAs encoded by the nuclear genes decline rapidly during protoplast isolation (Lett *et al.*, 1980; Vernet *et al.*, 1982), while these corresponding to the chloroplast genes continue to be expressed at a high level for at least two days (Criqui *et al.*, 1992a). They decline only later, when the chloroplast structure begin to be altered and starch begins to be accumulated in the plastid, i.e. when the transition from chloroplast to amyloplast is clearly detectable. Absence of coordination between the expression of the nuclear and chloroplast encoded genes has previously been observed during the shift from chloroplast to chromoplast. It is important to add that in spite of the presence in the freshly isolated protoplast of chloroplast LSU mRNAs at a level similar to the leaf, the corresponding polypeptide is not synthesized in the protoplasts, as shown by ^{35}S methionine labeling.

The same development is observed when leaf slices are cultivated in the protoplast medium or in water, indicating that neither plasmolysis nor maceration are necessary for the repression of the photosynthesis related nuclear genes. Sucrose (Criqui *et al.*, 1992a) and auxin (Takahashi *et al.*, 1989), if present in the maceration or culture medium, activate the decline of the mRNA level of the nuclear encoded photosynthesis-related genes. Nevertheless, the phenomenon, although slower, takes place in the absence of any metabolisable sugar or hormone suggesting that the rapid repression of these nuclear genes in mesophyll protoplasts is primarily induced by the mechanical wounding stress applied to the leaf prior to maceration.

Pathogenesis-Related Proteins

Tobacco plants reacting against TMV infection by hypersensitivity actively accumulate a small set of proteins which have been named Pathogenesis-related proteins (PR) (Van Loon, 1985). They are acidic, localized in the cell wall and the enzymatic activity of some of them has been characterized as hydrolases (Legrand *et al.* 1987; Kaufmann *et al.*, 1987) potentially able to destroy the cell wall of pathogens or saprophytes (Mauch *et al.*, 1988). They are generally considered to be defence proteins. More recently, it has been shown that another set of proteins is synthesized after TMV infection. They are basic, located in the vacuole and show considerable homologies with the acidic ones (Hooft van Huijsduijnen *et al.*, 1987). Consequently, they have been integrated into the PR proteins. Nevertheless, both groups are not regulated in the same manner: While acidic PRs are inducible by a limited number of stresses (pathogen infection, salicilate treament), basic PRs are also induced by wounding,

salt treatment (King *et al.*, 1988; Singh *et al.*, 1987) and are expressed without stress in different organs of the plant: at a very high level in mature roots and at a lower level in flowers and senescing leaves (Memelink *et al.*, 1990) and in *in vitro* cultivated callus (Mohnen *et al.*, 1985; Shinshi *et al.*, 1987). PR proteins are not detectable in seedlings, root tips or in heathly leaves which have just reached full expansion and are used for the isolation of tobacco protoplasts. The vacuolar basic PR proteins (glucanase, chitinases and osmotines) accumulate rapidly in protoplasts during culture. Nevertheless, acid PR are not detectable, with the exception of PR P and PR Q, two acidic chitinases which accumulate at a low level in protoplasts, and are released in low amounts into the culture medium (Grosset *et al.*, 1990). It is interesting to note that the same PR proteins are accumulated by callus cells and in leaf strips cultivated *in vitro*, i.e. the absence of most acidic PR is not related to the absence of the cell wall in protoplasts. In cells cultivated in liquid medium, PRs accumulate during the stationary phase but are undetectable during the growth phase. These proteins accumulate in extremely high amounts in leaf slices cultivated in water, at a level 5 to 10 time higher than in a medium compatible with callus formation. The presence of the osmoticum has little effect on this accumulation. Amongst medium compounds, auxin strongly reduces the mRNA accumulation of the PR proteins and of the corresponding mRNA, both in leaf slices and protoplasts (Grosset *et al.*, 1990).

Extensin

Extensins are glycoproteins having a very high hydroxyproline content (up to 45% of the aminoacids) Lamport (1965). They are located in the cell wall and seem to play a role both in cell growth and cell defence (Memelink *et al.*, 1987).

Using a carrot probe, Criqui (1992) showed the absence of transcript in the leaf. Protoplasts in culture first accumulate a 1.2 kb transcript and later a 1.4 kb transcript. Both transcripts are induced in leaf slices cultivated in the protoplast medium, indicating that the maceration is not responsible for the mRNA accumulation. In addition, these messenger RNAs accumulate to far higher levels when the leaf slices are cultivated in water, indicating that the high osmotic pressure of the culture medium is not necessary for the induction and that some elements of the culture medium inhibits the mRNA accumulation. Furthermore, both mRNAs are detectable in roots. Thus, both extensin transcripts are regulated in a way very similar to the PR mRNAs.

Ubiquitins

Ubiquitin is a small protein involved in the ATP-dependent selective degradation of cellular proteins (Hershko, 1983), maintenance of chromatin structure, regulation of gene expression, stress response (Fornace *et al.*, 1989), and ribosome biogenesis. Ubiquitin is synthesized as polyubiquitin containing two to seven motifs (Swindle *et al.*, 1988), or as a fusion protein comprising a ribosomal protein as a C-terminal extension of ubiquitin (CEP). The synthesis of this form has been associated with cell division and growth.

Using a human cDNA ubiquitin probe, Jamet *et al.* (1990) demonstrate the presence of at least four transcripts containing an ubiquitin sequence in *N. sylvestris*. Cloning shows that

the 0.8 kb transcript encodes a CEP (Genschik *et al.*, 1990). It is very abundantly expressed during the growth phase of tobacco cells and in growing tissues. This transcript is undetectable in the mature leaf, and present at a low level in protoplasts. Three other transcripts (1.3, 1.6, 1.9 kb) encode polyubiquitins. The 1.3 kb transcript is present in stressed and dividing tissues, the 1.6 kb transcript is induced early by protoplast isolation but not by other stresses (heat-shock, $HgCl_2$), while the 1.9 kd transcript is present in both protoplasts and stressed tissues (Criqui, 1991, J. Fleck personnal communication).

HMG-CoA-Reductase and Putative Glutathione Peroxidase

Criqui *et al.* (1993a) prepared a cDNA library from *N. sylvestris* protoplasts isolated by an overnight maceration followed by six hours culture in a medium compatible with protoplast division. They selected two clones corresponding to mRNA not detectable in the leaf, expressed both in protoplasts and in leaf slices cultivated in the protoplast medium.

The first clone shows a very high homology to an *Arabidopsis thaliana* HMG-CoA-reductase (70% identity of the nucleotide sequences). This enzyme catalyses the first reaction of the isoprenoid pathway, from which various key cellular compounds are synthesized (mevalonic acid, sterols, carotenoids...) (Bach, 1987; Yang *et al.*, 1991). Its mRNA is present at a low level in apex, anthers and roots but surprisingly absent from seedlings and young leaves. The absence of this mRNA in these rapidly growing organs suggests that other mRNAs encode HMG-Co-reductases. The messenger accumulates at a high level in protoplasts during culture and is well expressed in cell suspensions. It is also induced in leaf slices cultivated *in vitro* in the protoplast medium. $HgCl_2$ vaporisation on unwounded leaves also induces the mRNA at a high level.

The second clone shows 33% identity with animal seleno glutathione peroxidases, which are considered as key detoxification enzymes able to trap hydrogen and lipid peroxides (Ischii, 1987). Although the enzymatic activity of the encoded protein is not definitly established, due to the relatively low sequence homology, the pattern of mRNA accumulation has been analyzed in details: It is expressed at a high level in seedlings but not in mature organs (leaves, stem, roots). It is expressed very early in fresly isolated protoplasts, and then decline in culture. It is also induced in leaf strips, or in unwounded leaf by $HgCl_2$ vaporisation. This mRNA is also detectable in suspension cells.

Anionic Peroxidase, Serine Protease Inhibitor 1, 6P50

Criqui *et al.* (1993b) isolated from their *N. sylvestris* library three cDNA clones not expressed in the leaf, expressed early in protoplasts and only at a low level in leaf slices cultivated in the protoplast medium.

The first clone encodes one of the multiple tobacco peroxidases. *In vitro* peroxidases are able to oxidize guaiacol or other synthetic substrates in the presence of hydrogen peroxide, but the cellular substrate is generally unknown. They are implicated in auxin degradation, lignin and suberin formation, defence against pathogens and possibly germination and root formation (Lagrimini and Rothstein, 1987; Lagrimini *et al.*, 1990). This mRNA is detectable in roots but not in other organs.

The second clone encodes a serine protease inhibitor 1, a member of one of the numerous groups of plant proteinase inhibitors. Inhibitors 1 have been described in various plants during senescence or pathogenicity, but not after wounding (Ryan, 1989). They are supposed to inhibit pathogen proteases rather than plant proteases and to be defense peptides. Nevertheless, participation of proteinase inhibitors in development cannot be excluded, especially in the case of the peptide encoded by the clone isolated from the protoplast library: It is devoid of the pro-sequence supposed to be necessary for processing into the vacuole and may remain in the cytoplasm. The mRNA is detectable in roots but not in other organs.

The third clone presents an open reading frame having no homolog in the data banks, but contains a motif which may acts as a nuclear localization signal. The corresponding mRNA is not detectable on Northern blots in any tobacco tissue.

The three mRNAs are rapidly induced in protoplasts during isolation and they disappear rapidly (1 to 4 hours) in culture. Although the clones were isolated for their low expression in wounded leaves cultivated in protoplast medium, the mRNA is already induced by wounding, but at a low level. In contrast, they are not induced by $HgCl_2$ treatment, heat shock, virus or *Agrobacterium* infection.

Tnt1 Retrotransposon

Grandbastien *et al.* (1989) isolated from *N. tabacum* a retrotransposon closely related to the copia element. The 5.2 kb element contains two perfect 610 bp long terminal repeats (LTR) at the borders and a single 3984 bp open reading frame encoding a gag-pol functional domain. A 6.5 kb RNA present in leaves, petals and roots as well as in mesophyll protoplasts has been interpreted as a chimeric plant/retrotransposon transcript. A 5.2 kb RNA, i.e. the expected size for the transposition form, is detectable in roots, not in the leaf, but is strongly induced in freshly isolated protoplasts and then drops rapidly in culture (Pouteau *et al.*, 1991). It is not detectable in wounded leaf but is present at a low level in suspension cells. In addition, tobacco protoplasts isolated from a transgenic plant containing a LTR/GUS construction present very high GUS activity

Par A and Par B

Takahashi *et al.* (1989) have constructed a cDNA library from tobacco mesophyll protoplasts cultivated *in vitro* for 24 hours in complete medium, then screened the library with cDNA prepared with 24-hour old protoplasts cultivated with or without the auxin 2,4-D. The first clone that they isolated, par A, presents an open reading frame which shows low homology (22%) to a stringent starvation protein of *E. coli* that binds to RNA polymerase and better homology to three auxin regulated genes isolated from *N. plumbaginifolia*, soybean and potato (Dominov *et al.*, 1992). The function of the encoded proteins is not known. The mRNA is not detectable in leaf, and in other organs of the plant except young seedlings and roots (Takahashi *et al.*, 1992). It is not detectable in freshly isolated protoplasts or in protoplasts cultivated in the absence of auxin, but is induced rapidly (30 mn) by auxin treatment of 24-hour old protoplasts. It is not induced by cytokinin, gibberellin or abscissic acid, but is induced by $CdCl_2$ or cycloheximide, suggesting that the par A gene belongs to a superinduction gene family.

The second gene isolated (Takahashi and Nagata, 1992) encodes a glutathione S-transferase as shown by sequence homology of the cDNA and protein production in *E. coli*. This enzymatic activity has been associated with detoxification, but also with cell proliferation in animal cells (Suthanthiran *et al.*, 1990). The time course of mRNA accumulation in protoplasts is very similar to that of par A. Information on par B expression in tobacco plant is not available presently.

Thioredoxins and a Homolog to a Tumor Suppressor

Marty *et al.* (1993) prepared a cDNA library from tobacco cells cultivated *in vitro* and isolated 8 clones encoding mRNAs not detectable in the leaf and expressed at a higher level in tobacco cells during the growth phase than during the stationary phase, with the aim of isolating sequences related to cell proliferation. They then follow mRNA accumulation during leaf protoplast isolation and culture. Surprisingly, out of 8 clones, 7 are expressed very early and at a high level. Two of them are abundantly expressed during the short term isolation procedure (3 hours) and continue to be expressed in culture. The first clone encodes a cytoplasmic thioredoxin (Thio h1) (Marty and Meyer, 1991), as shown by sequence homology to animal thioredoxins, and expression in *E. coli* (Marinho *et al.*, in preparation). Thioredoxins are small proteins with the conserved disulfide site WCGPC. The oxidized form contains a disulfide bridge, while the reduced form is a powerful oxidoreductase (Flamigni *et al.*, 1989), able to reduce the disulfide bridges of target proteins. In higher plants, two chloroplast thioredoxins regulate the light/dark reactions (Jacquot, 1984; Wolosiuk and Buchanan 1977), while in animals (Grippo *et al.*, 1985; Tagaya *et al.*, 1989) and yeast (Müller, 1991) cytoplasmic thioredoxins are associated with proliferation and control of the cell cycle (Holmgren, 1989).

The second clone encodes a protein which shows strong homology to a suppressor of a human Wilm's tumor (Dowdy *et al.*, 1991). The presence of the similar mRNAs in human brain, human mammary gland, mouse, rice endosperm and *Arabidopsis* (Rivera-Madrid *et al.*, 1993), as well as the very high conservation of the sequence from man to plants suggest a more general role. The mRNA is detectable mostly in rapidly-growing tissues, but not in mature tissues.

Both mRNAs are undetectable in tobacco leaf, but accumulate at a high level during short term isolation (3 hours). This high level is maintained in culture, but only in the presence of sucrose in the culture medium.

More recently, it has been shown than tobacco contains at least one other thioredoxin gene (Thio h2) (Brugidou *et al.*, 1993). Both genes are expressed in growing tissues but not in mature tissues. Thio h2 mRNA does not accumulate during protoplast isolation, but only later, after 4 hours in culture (Marty *et al.*, 1992).

Ribosomal Proteins, Elongation Factor 1α, and Cyclophilin

The same screening allowed the isolation of 6 additional clones. Two encode the cytoplasmic ribosomal proteins RL2 and S4 and one encodes a translation factor 1α (Liboz *et al.*, 1989; Pokalsky *et al.*, 1989; Ursin *et al.*, 1991). Although ribosome biosynthesis is clearly necessary for cell proliferation, the selection of this type of clone during this screening was unexpected because it indicates that these ribosome compounds are not synthe-

sized in the mature leaf. In fact it appears that ribosome synthesis in plants is coupled to cellular proliferation and is not detectable in mature tissues (Lebrun and Freyssinet, 1991), even those which actively synthesize proteins, like seeds during storage protein accumulation (Marty and Meyer, 1992).

A further clone encodes a cyclophilin, a peptidyl-prolyl cis-trans isomerase thus able to modify the conformation of target proteins (Bachinger, 1987). Cyclophilins are associated with cell proliferation in human t lymphocytes (Fischer *et al.*, 1989) and detected in growing plant organs (Gasser *et al.*, 1990; Marivet *et al.*, 1992).

A fifth clone presents no homology with the protein data banks. All five mRNAs are abundant in rapidly-growing tissues, but not in mature organs. They are detectable in protoplasts after 4 hours in culture, or in the maceration solution if the treatment is applied for a longer time. In contrast, if freshly isolated protoplasts are cultivated in $KCl+MgSO_4$, the mRNAs do not become detectable. Thus it appears that something common to the maceration medium and the culture medium is necessary for the induction of these genes. In contrast to thioredoxin and to the homolog of the Wilm's tumor suppressor, sucrose is not necessary for this induction (Marty *et al.*, 1993). No mRNA corresponding to the sixth clone can be detected in protoplasts.

Histone H3 and H4

Histone H3 (Takahashi *et al.*, 1989) and H4 (Marty *et al.*, 1992) probes (Chaboute *et al.*, 1987) have been used as markers of the S phase. In both cases Northern blots with protoplast mRNAs present very low signals even with protoplasts actively incorporating ^{35}S methionine.

DISCUSSION

Recently our knowledge on genes expressed early after mesophyll protoplast isolation have increased significantly, revealing the expression, among others, of some genes which were not suspected to be present in plants some years ago. In fact this situation results from progress in cDNA cloning, sequencing and data base analysis, and is also common in other fields of plant physiology. It is to be hoped that more mRNA transcripts expressed very early will be characterized because this constitutes an important basis for the understanding of the mechanisms and inducers implicated in dedifferentiation. In spite of the relatively limited number of mRNAs presently characterized, the nature of the proteins that they encode deserves some comments: Clearly no gene directly related to mitosis has been shown to be expressed in protoplasts, although plant cDNAs encoding cdc2 (Feiler and Jacobs, 1991; Ferreira *et al.*, 1991) and cyclins (Hemerly *et al.*, 1992) have been isolated in different labs. In fact H3 and H4 histones mRNAs are present only at a low level in mesophyll protoplasts.

There is no doubt that genes controlling mitosis or encoding structural proteins necessary for mitosis (histone, DNA polymerases...) have to be expressed in protoplasts for the progression through the cell cycle, but we can recall that the first cell cycle associated with dedifferentiation is very long. In a population of tobacco mesophyll protoplasts, the first

cells divide after 48 hours, but most protoplasts undergo their first mitosis far later, only after 4 to 5 days. This should be compared to the 20-24 hour cell cycle of meristematic or suspension cells. Consequently, a low level of mRNAs encoding mitosis related-proteins is probably sufficient. Even the auxin dependent gene parA is clearly not directly related to mitosis: it is not expressed at a high level in shoot tips and young leaves, organs in which cells actively divide. This is coherent with the pleiotropic effect of auxin which is not only necessary for the progression into the cell cycle, but also plays roles in cell wall regeneration, vacuolisation, cytoplasmic activity and detoxification of the medium. The growth-related genes reported in this review are overexpressed in protoplasts, when compared with proliferating cells. This clearly shows that they are not directly implicated in cell cycle progression. In fact most genes expressed during proliferation are not implicated in mitosis, but produce basal cellular compounds: this is clearly illustrated by the active expression of ribosomal protein and elongation factor genes.

Amongst the twelve genes presently characterized three, selected by independent screenings are related to redox reactions. Although this may be fortuitous, it is also possible that this indicates that this relatively neglected aspect of cell metabolism plays a key role in cell development both in regulating protein activity (thioredoxin) and trapping peroxide radicals (glutathione S-transferase, glutathione peroxidase) produced by the active cell metabolism.

One of the long term goals is to characterize the mediators which induce the mesophyll cell to dedifferentiate. The first step is to determine the events in protoplast isolation which lead to the synthesis of new mRNAs: PR protein and extensin mRNAs are induced in leaf slices (1-2 mm wide) at approximately the same level as in protoplasts, but not or only at a low level in large leaf explants. This is coherent with the participation of an inducer liberated by wounding which migrates approximately one mm in the mesophyll, i.e. 20-30 cell layers. In addition, these mRNAs are far more abundant when leaf slices are maintained in a medium incompatible with the induction of mitosis in the explant. Growth-related mRNAs are induced at a level 2-5 times lower in leaf slices than in protoplasts. This is compatible with an induction in cells near the wound, up to 1 to 2 cell layers away. These genes are not induced in a medium incompatible with growth. A screening has been directed to the characterization of mRNAs mostly abundant in protoplasts, but not in leaf slices cultivated in the protoplast medium. Using the resulting clones, it has later been shown that these mRNAs are already induced in the leaf slices, but at a far lower level than in protoplasts. It is well known that when leaf slices are cultivated, callus formation takes place at the level of the wound and begins in a few spots. Thus this difference in the mRNA levels is compatible with the induction of these genes in a few cells at the wounded surface of the explant. Clearly *in situ* hybridization studies are needed to confirm this interpretation. Nevertheless, wounding has been found to be the inducing step for most of the genes expressed early in protoplasts, although additional conditions in the composition of the culture medium are required for some of them. The induction of Tnt1 is one exception, as it has been attributed to the maceration (Pouteau *et al.*, 1991). In fact it is likely that, due to the particular situation of protoplasts, not all genes expressed early in these cells are related to dedifferentiation, and that some of them may results from the high osmotic pressure of the culture medium (Fleck *et al.*, 1982), from the use of crude enzymes or from the maceration.

Nevertheless, the studies on large leaf explants and slices very clearly show that most genes expressed early in protoplasts are induced by wounding and that the original wound signal is probably relayed by secondary messangers which present different mobilities in plant tissues. Wound-induced genes have been described (Hilmann *et al.*, 1992; Pena-Cortes *et al.*, 1988; Stanford *et al.*, 1990; Weissand and Bevan, 1991) and for some of them the promoter has been analyzed (Logemann *et al.*, 1989). However, mediators have been characterized only in the case of proteinase inhibitor II. After wounding of tomato plants proteinase inhibitors II are synthesized in a very systemic way: wounding one leaf induces the synthesis of proteinase inhibitor II in other leaves (Ryan, 1987). It was first shown that oligosaccharide wall compounds are released by wounding and have the potential to induce the systemic response, but that they do not migrate into the plant, suggesting the induction of a second messenger (Ryan and An, 1988). This has been recently shown to be a protein, which has been named systemin. Thus, this model predicts that wounding releases non-migrating oligosaccharides which induce systemin gene expression, systemin synthesis and secretion in cells near the wound (Pearche *et al.*, 1991). In addition, another compound, jasmonic acid, seems to be a further intermediate in the induction of proteinases inhibitors II in the target cells (Farmer and Ryan, 1992). This illustrates very well how complex a plant response to a particular effector can be. The complexity and diversity of the reactions of plants to wounding can probably be better understood from a physiological and ecological point of view. Plants do not have the possibility to move to protect themself against aggressions, but possess different defence strategies which are complementary. Wounding frequently results from insect feeding. After a first injury some plants resist a possible invasion by systemic synthesis of proteinase inhibitors that make the rest of the plant inedible. In addition, the open wound is a very favourable site for pathogens and even for saprophyte entry. Plants react by a local or nearly local induction of defence proteins which block the growth of parasites or by necrosis, the dead cells constituting a medium which is not adapted to the growth of parasites. Finally, if the physiological condition of the tissues is adequate, some cells recover their proliferation potential, form a wound callus which repairs the damage, even when this is considerable (i.e. grafting), or form new meristems if necessary (cuttings, decapitated plants). All the genes playing a role in these different processes could be described as wound-inducible, but are nevertheless regulated in very different ways and probably have no common structures in their promoters.

Our present knowledge of gene expression in mesophyll protoplasts shows that at least defence and proliferation-related genes are expressed early and continue to be expressed in *in vitro* callus. This shows the strong similarity between *in planta* healing callus and *in vitro* cultivated callus. In addition to common gene expression, *in vitro* callus has most of the characteristics of healing callus, including the ability to form meristems and there is a striking parallel between the ability of some tissues to form a healing callus after wounding and to proliferate *in vitro* (very high potential of the cambium of dicots, extremely low potential of the mature tissues of cereals). Thus, we propose that the analysis of early gene expression in mesophyll protoplasts will provide information relevant to general processes of plant development, including dedifferentiation and wound reactions, and possibly provide solutions which will help to overcome the inability (Siminis *et al.*, 1992) of some cells to

dedifferentiate and form callus *in vitro* for example pollen or mature cells in cereals (Hahne *et al.*, 1989; Wang *et al.*, 1989).

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