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Identification of a novel transcriptional activator involved in plastid-nucleus communication during the plant response to stress

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Résumé

Les plastides et mitochondries sont dérivées d'endosymbionts prokaryotiques. Au cours de l'évolution, une grande partie de leurs génomes a été transférée au noyau. Ce dernier contrôle donc le protéome des organelles de la cellule. Cela permet la coordination des activités métaboliques et facilite l'adaptation de la plante aux conditions changeantes de l'environnement. La signalisation inverse, des organelles au noyau, est peu caractérisée. Des signaux chloroplastiques, tels que les précurseurs de la chlorophylle et certains intermédiaires oxido-réducteurs affectent les activités du noyau par des mécanismes inconnus. Nous présentons ici le clonage du gène codant pour p24, un nouvel activateur transcriptionel des gènes de défense dans le noyau. Il est donc surprenant que p24 se retrouve aussi dans les chloroplastes de la plupart des cellules, où il lie l'ADN de façon séquence-spécifique et affecte l'expression d'un gène impliqué dans la photosynthèse. p24 affecte également le transport linéaire et cyclique d'électrons lors de la photosynthèse. De plus, l'expression du gène nucléaire *Fed1* est contrôlée par l'effet de p24 sur la photosynthèse. Nous concluons que p24 produit un signal photosynthétique qui se traduit par des changements d'expression de gènes nucléaires.

La localisation nucléaire de p24 semble être contrôlée par un programme développemental induit par l'hormone cytokinine. Les cytokinines, tout comme les stress biotiques et abiotiques, induisent l'assimilation des sucres solubles par les tissus « puits ». Cela est en accord avec l'observation de p24 dans le noyau de cellules de racines, qui doivent obtenir leurs sucres de tissus photosynthétiques qui agissent comme « sources ». Le mécanisme pour la double localisation de p24 n'est pas clair, mais pourrait dépendre d'extension des chloroplastes, les stromules. Nous démontrons ici que ces stromules sont associés avec le réticulum endoplasmique.

En conclusion, la double localisation de p24 permet la coordination des activités de défense des différents compartiments de la cellule, et aussi entre tissus sources et tissus puits. p24 joue probablement un rôle important dans l'orchestration de la réponse de défense à travers toute la plante.

Mots clés : pomme de terre, *Phytophthora infestans*, plastides, stromules, double localisation, PR-10a, source, puit, cytokinine, photosynthèse

Summary

Plastids and mitochondria have evolved from prokaryotic endosymbionts. They have in the process surrendered a large portion of their genomes to the nucleus, which then exerts control over the protein complement in plastids and mitochondria. This is required for the cell to coordinate its biochemical and physiological activities and to adapt to constantly changing conditions. The reverse signalling, from organelles to the nucleus, is not as clearly understood. In plants, chloroplasts signal the nucleus via their redox state and chlorophyll precursors, through unknown mechanisms. Here, we report on the cloning of the gene coding for p24, a novel plant transcriptional activator of defence genes in the nucleus. Strikingly, p24 is most often present in the chloroplast where it binds DNA in a sequence-specific manner and drives stress-dependent photosynthetic gene expression. p24 also affects linear and cyclic photosynthetic electron flow (PEF). In addition, the expression of the nuclear gene *Fed1*, known to be controlled by PEF, is also affected by altered p24 levels in transgenic plants. We conclude that chloroplast p24 induces a photosynthetic signal that affects nuclear gene expression.

Interestingly, a developmental program induced by the plant hormone cytokinin causes p24 nuclear localization in cultured leaf cells. Cytokinins, as well as biotic and abiotic stress, are known to induce sink activity. This is consistent with the observation of nuclear p24 in the roots, which must receive sugars from photosynthetic source tissues. The mechanism for p24 dual localization is not clear, but may involve translocation through plastid extensions known as stromules, which are shown here to associate with the endoplasmic reticulum.

We speculate that dual-localization of p24 allows for tight coordination of gene expression in plastids and the nucleus, and also between sink and source tissues. p24 is likely an important player in orchestrating the whole-plant adaptation to stress.

Key words: potato, *Phytophthora infestans*, plastids, stromules, dual localization, PR-10a, source, sink, cytokinin, photosynthesis

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List of Abbreviations

AIF	Apoptosis inducing factor
Avr	Avirulence
BAC	Bacterial artificial chromosome
BAP	6-benzylaminopurine
bZIP	Basic leucine zipper
CaMV	Cauliflower mosaic virus
CC	Coiled-coil
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CS	Coding strand
CTAB	Hexadecyltrimethylammonium bromide
CWInv	Cell wall (extracellular) invertase
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
EEE	Excess excitation energy
EMSA	Electromobility shift assay
ER	Endoplasmic reticulum
ERE	Elicitor-response element
ERF	Ethylene response factor
ETR	Electron transport rate
EST	Express sequenced tag
FPLC	Fast protein liquid chromatography
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
GTF	General transcription factor
GUS	β-glucuronidase
HR	Hypersensitive response
IPTG	isopropyl-β-D-thiogalactopyranoside
JA	Jasmonic acid
LCS	Leica confocal software
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LSCM	Laser-scanning confocal microscopy
LSD1	Lesion simulating disease 1
MAPK	Mitogen-activated protein kinase
MLS	Mitochondrial localization signal
NBS	Nucleotide-binding site
NCS	Non-coding strand

NDGA	Nordihydroguaiaretic acid
NDH	NAD(P)H dehydrogenase
NEP	Nuclear-encoded polymerase
NLS	Nuclear localization signal
NO	Nitric oxide
NPP1	Necrosis-inducing *Phytophthora* protein 1
NPQ	Non-photochemical quenching
PAM	Pulse amplitude modulated
PAM	Point accepted mutations
PAMP	Pathogen-associated molecular pattern
PB	p24-binding site
PBF-2	PR-10a binding factor 2
PCD	Programmed cell death
PCR	Polymerase chain reaction
PEP	Plastid-encoded polymerase
PEF	Photosynthetic electron flow
PEG	Polyethylene glycol
PKC	Protein kinase C
PS	Photosystem
R	Resistance
ROI	Region of interest
ROS	Reactive oxygen species
SA	Salicylic acid
SABP	SA-binding protein
SAGE	Serial analysis of gene expression
SAR	Systemic acquired resistance
SIPK	SA-induced protein kinase
SPP	Stroma processing peptidase
Tic	Translocon at the inner membrane of chloroplasts
TIR	Toll and interleukin-1 receptor
TMV	Tobacco mosaic virus
Toc	Translocon at the outer membrane of chloroplasts
VIGS	Virus-induced gene silencing
VPE	Vacuolar processing enzyme
WIPK	Wound-induced protein kinase
WT	Wild-type

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Preface

The topic of this thesis was, initially, the transcriptional activation of the defence response. With new advances in this field however, it has become obvious that the plant response to pathogens is intimately linked to all other plant functions, including growth and development. This is illustrated by many of the experimental results presented here.

The format of this thesis has therefore been adjusted to reflect the breadth and diversity of topics covered. As such, a short general introduction precedes three chapters that each present a detailed introduction to the topic(s) addressed, methods, results and discussion. A final discussion chapter attempts to link the three topics and to put them in the wider perspective of plant biology as a whole.

The results presented in Chapter II (the cloning of the *p24* gene) have been published in *Plant Cell* (2000) **12**:1477-1489. A second publication on the results presented in Chapter III is currently in preparation.

Chapter I

Introduction

The Plant Defence Response

Plants are sessile organisms and, as such, they must have elaborate adaptation mechanisms to respond to an ever changing environment from which they cannot escape. These mechanisms are closely interrelated with systems to evade or resist pathogen and herbivore attack.

Plants defend themselves using both pre-formed and inducible mechanisms. The constitutively synthesized toxins produced by a few species, the wax layer covering some leaves, as well as the cell wall surrounding plant cells serve as effective barriers for a large number of potential invaders. Some pathogens do breach these barriers however and, in response, plants activate both general (non-host) and host-specific resistance mechanisms. While, in animals, these two responses are separate and correspond to innate and adaptive immunity respectively, plants do not have adaptive defence responses. As a consequence, there is considerable overlap between host and non-host resistance effectors in plant cells [1].

Non-Host resistance

Non-host resistance can be passive, such as the deposition of callose at the site of pathogen entry, but is most often induced. Induced non-host resistance in plants is similar to animal innate immunity, which activates pathogen resistance when the host recognizes general pathogen-associated molecular patterns (PAMPs) [1]. The latter are unique to pathogens and are indispensable for pathogenicity [2]. Surface-derived structural molecules from plant pathogens, such as fungal cell wall components (chitin, glucan, protein and glycoprotein), bacterial lipopolysaccharide (LPS) and flagellin, induce defence responses in a wide range of plant species [3-5]. Another example is NPP1 (necrosis-inducing *Phytophthora* protein 1), a *Phytophthora* cell wall protein, that is a member of a protein family that is widespread among oomycetes, fungi and bacteria, and has elicitor activity in dicots [6]. Because of their characteristics and their wide host range, pathogen elicitors are therefore conceptually similar to PAMPs [1].

Plant cell wall degradation products, such as oligogalacturonides, resulting from pathogen activity also act as strong elicitors [7]. In addition, the pathogen enzymes themselves are often recognized by the plant. A recent example is the surface transglutaminase GP42 from *Phytophthora* spp. oomycetes. Pep13, a 13 amino acid peptide that acts as a strong elicitor in plants of the Solanaceae family (such as potato and tomato), lies in the active site of GP42 [8]. Interestingly, Pep13 is recognized by potato, yet this plant is highly susceptible to *Phytophthora infestans* [8]. The fact that Pep13 recognition is not sufficient for resistance therefore calls into question the biological relevance of elicitor detection in plant defence. It could be however that pathogens have the ability to suppress basic non-host resistance.

Host-specific resistance

In contrast, host-specific resistance results from the recognition of a single gene product from the pathogen (avirulence gene, *Avr*) by a single resistance gene from the plant (*R* gene). This "gene-for-gene" theory stems from the pioneering experiments of Flor [9] on the flax-rust interaction and states that the presence of a gene in one population depends on the presence of a corresponding gene in another population. The interaction of these two genes produces a single phenotype (disease resistance, known as an incompatible reaction) [10]. The absence of either gene in the plant-pathogen pair causes disease (known as a compatible reaction). Therefore, there appears to be a constant evolutionarily battle between plants and their pathogens. In nature however, disease is rare and, when it occurs, it rarely kills the plant [11]. In fact, it has been hypothesized that the gene-for-gene type of interaction allows the long-term survival of both the plant and the pathogen species [12]. Still, the fact that pathogens maintain *Avr* genes in their genome is puzzling. In practice however, most *Avr* genes are actually virulence factors in the absence of the corresponding plant *R* gene, and there is mounting evidence that these virulence determinants are able to suppress the plant defence response [1]. For example, the pathogen *Pseudomonas syringae* pv. Tomato DC3000 uses a type III secretion system to inject virulence effectors in plant cells. These proteins induce a form of programmed cell death (PCD, known as the hypersensitive response, HR) in resistant plants. It was shown that mutants for 6 proteins (HopPtoE, AvrPphEPto, AvrPpiB1Pto, AvrPtoB, HopPtoF and HopPtoG) elicited a stronger HR,

suggesting that these proteins are inhibitors of cell death [13, 14]. In fact, these bacterial effectors were also found to inhibit the ability of the pro-apoptotic protein Bax to induce PCD in plants and yeast [14]. The Delta CEL mutation in *P. syringae* and the dspA/E mutation in *Erwinia amylovora* are also known to be impaired in virulence on their *Arabidopsis* and apple host plants respectively. More specifically, these mutations elicit increased cell wall-based defences and are not able to cause normal disease necrosis in plants [15]. Interestingly, these mutant phenotypes are dependent on the presence of the signalling molecule salicylic acid (SA) in the plant and, in *P. syringae*, the hopPtoM and AvrE proteins were specifically found to be responsible for the suppression of SA-mediated defences [15]. SA is an important mediator of the plant defence response, and the ability to suppress SA-induced defences represents a significant advantage for pathogens. The widespread conservation of pathogen effectors and the large number of these effectors involved in suppressing the plant defence response suggests that this is an important strategy for infection of host plants.

R genes

To counter these virulence genes, plants have evolved *R* genes that recognize and neutralize the activity of their corresponding *Avr* gene. *R* genes belong mostly to two structural classes: the nucleotide-binding site – leucine-rich repeat (NBS-LRR) class, and the receptor LRR-kinases [reviewed in 16]. While LRR-kinases have been also associated with normal plant development and hormone perception [17, 18], NBS-LRR proteins have so far only been linked to plant immunity [16]. In the model plant *Arabidopsis thaliana*, there are 149 NBS-LRR genes [19]. NBS-LRR proteins have distinct N-terminal domains. These can be either a coiled-coil (CC) domain, or a domain sharing homology with the Drosophila TOLL and mammalian IL-1 receptors (TIR). This suggests certain similarities between plant defence and animal innate immunity. In support for this hypothesis, the NBS domain shares some homology with that of animal pro-apoptotic proteins such as APAF-1 [20, 21].

Of all these domains, however, mutational analysis indicates that the LRR domain confers recognition specificity to the R-genes [reviewed in 22]. Given the gene-for-gene hypothesis, it appears logical that the R proteins act as receptors and that they interact directly with pathogen avirulence proteins. It has indeed been found that AvrPi-ta from the rice blast pathogen

Magnaporthe grisea can bind directly to the LRR domain in the rice R protein Pi-ta [23]. This is the exception rather than the rule however and, in most plant-pathogen interaction, no interaction can be observed between the corresponding *R* and *Avr* gene products [1]. Alternative hypotheses are therefore needed to explain the role of *R* gene products in plant disease resistance.

The guard hypothesis

It has recently been proposed that R gene products sense the effect of the Avr proteins rather than the proteins themselves [reviewed in 24]. As such, R proteins would act as "guards" for the plant targets of the pathogen Avr factors and would detect any interference with the host protein functions.

Strong support for the guard hypothesis comes from the observation that the *Arabidopsis* RPS5 NBS-LRR protein requires another host protein, the kinase PBS1, to function [25]. Interestingly, the pathogen protein AvrPphB is a self-cleaving cysteine protease [26] that can also cleave PBS1 [27]. The kinase activity and cleavage of PBS1 are both required for RPS5 activation and signalling [27]. This suggests that RPS5 can "sense" the effect of AvrPphB in the cell. It would be interesting to determine the role of PBS1 proteolysis in virulence, in the absence of the RPS5 resistance gene.

The discovery of the RIN4 protein from *Arabidopsis* has also supported the guard hypothesis. RIN4 forms a complex with the resistance proteins RPM1 [28] and RPS2 [29, 30]. RPM1 confers resistance to *P. syringae* harbouring the *AvrB* and *AvrRpm1* genes [31], while RPS2 recognizes *AvrRpt2* [32, 33]. Interestingly, AvrB and AvrRpm1 cause the phosphorylation of RIN4 and activation of RPM1 [22]. On the other hand, AvrRpt2 causes the degradation of RIN4 [29, 30]. This interferes with the function of RPM1, but activates RPS2. According to the guard hypothesis, RIN4 would therefore be the target of bacterial virulence proteins and is the "guardee" of both RPS2 and RPM1 though, once again, a role for RIN4 in virulence has yet to be demonstrated.

Two recent studies have shown that RIN4 is not the only target of AvrRpm1 and AvrRpt2 because these effectors are able to promote virulence independently of RIN4 [34, 35]. Thus, RIN4 may be important for disease resistance (bacterial avirulence), but not for bacterial virulence. This highlights

the fact that there is still much to do to decipher the role of resistance protein complexes in virulence and avirulence.

Downstream responses

Despite the varied nature of plant-pathogen interactions and the presence of pathogen-specific recognition mechanisms in the plant, it was suggested more than a decade ago that differences in downstream defence responses between a resistant and susceptible plant are mostly quantitative/kinetic rather than qualitative in nature [36]. This was confirmed by a recent large scale analysis of gene expression during compatible and incompatible interactions between *Arabidopsis* and the pathogen *P. syringae* [37]. In this study, the expression of roughly 8000 genes was monitored using an oligomicroarray approach in which each gene is represented by a set of 16 to 20 oligonucleotides. Interestingly, approximately 2000 genes (25%) showed reproducible and significant (at least 2 fold difference as compared to control) expression level changes in at least one of the interactions studied. This strongly suggests that plant defence entails comprehensive reprogramming of cellular metabolism. More importantly, the genes affected in basal resistance overlapped significantly with those involved in R-gene specific resistance. The gene expression modifications were only delayed in the compatible interaction. Both the shape and amplitude of the gene expression profiles 30 h after infection with a low-dose of a compatible pathogen were similar to the profiles obtained 9hrs post-infection in incompatible interactions. It was apparent however that gene expression resulting from incompatible interactions was more robust and less sensitive to biological variation than compatible interactions. Surprisingly, even when genetically separable R-gene pathways (*RPS2* and *RPM1*) were analyzed, the gene expression patterns were highly correlated (93% at 9 hrs post-infection). This suggests that different signalisation pathways must converge onto a single pathway responsible for the changes in gene expression. The authors propose a model in which perception by distinct R-genes modulates the amplitude of a common signalling pathway [37]. This is in agreement with the fact that RPS2 responses are generally slightly slower than those mediated by RPM1, despite having the same profile.

RPS2 responses (but not RPM1 responses) are strongly suppressed by mutations in the *NDR1* gene, and by the presence of the *NahG* transgene

that encodes salicylate hydroxylase and prevents the accumulation of SA [37]. Of note, *ndr1* mutants also have defects in SA accumulation [38]. In other words, RPS2 responses are more sensitive to SA than RPM1. Yet these two R genes utilize different pathways to obtain similar results, only with different intensities. Previous observations had suggested a signal amplification loop controlled by SA [39]. Endogenous SA at physiological concentrations (50 μM) is known to amplify the expression of defence genes in the presence of a pathogen signal, independently of de novo protein synthesis [39]. It is therefore possible that, for RPS2, SA is important in amplifying the input signal so that resistance will occur, whereas the RPM1 signal is already strong enough to elicit resistance on its own [37].

Transcriptional activation of defence genes

The proposed common resistance signalling pathway leads to a reprogramming of gene expression in response to pathogen attack [37]. The transcription factors responsible for defence gene expression are highly sought after and have begun to be identified.

The WRKY transcription factors were first assigned a defence role in parsley [40] and represent the largest family of defence transcription factors identified so far with up to 100 representatives in *Arabidopsis* [41]. Not all are involved in defence however. WRKY transcription factors have been implicated in senescence, trichome development, root cell maturation, gibberellin signalling, and flower development [42]. All members of this protein family have a conserved DNA-binding domain characterized by the invariant amino acids W-R-K-Y-G-Q-K, hence their name. The structure of this domain was recently elucidated and reveals a novel zinc and DNA binding structure consisting of a four-stranded beta-sheet with a zinc binding pocket formed by conserved Cys/His residues located at one end of the beta-sheet [43]. WRKY proteins bind the consensus DNA sequence (C/T)TGAC(T/C), known as a W-box, and different family members act as activators and repressors of transcription [41].

Despite their varied roles, WRKY factors are strongly associated with the stress response. A change in the expression of 49 out of 72 tested *Arabidopsis* WRKY genes occurred in response to SA treatment or infection by the bacterial pathogen *P. syringae* [44]. In tobacco, several WRKY genes are induced in response to treatment with SA, fungal elicitors or H_2O_2 and

following infection with tobacco mosaic virus (TMV) or bacteria [45-48]. Finally, a gain-of-function mutation in a mitogen-activated protein kinase (MAPK) that acts upstream of SA-induced (SIPK) and wound-induced (WIPK) protein kinases caused the transcriptional activation of four WRKY genes and an increase in protein-binding to W-boxes in vitro [49].

There is also functional evidence for the involvement of WRKY genes in defence. The overexpression of *AtWRKY29* in *Arabidopsis* provided increased resistance to virulent *P. syringae* bacteria [50]. Furthermore, virus-induced gene silencing (VIGS) of three WRKY genes compromised resistance to TMV conferred by the *N* resistance gene [51]. The expression of the gene coding for the plant defence regulator NPR1 is itself controlled by WRKY transcription factors [52]. Finally, the protein WRKY70 was found to be a node of convergence for defence signalling induced by two important molecules: SA and jasmonic acid (JA) [53]. There is a known antagonism between these two pathways [54] and it was found that WRKY70 activates SA-mediated responses, while it inhibits JA signalling [53].

A definitive genetic link between WRKY proteins and the defence response came from the cloning of the *RRS1* gene responsible for resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* [55]. This unique gene codes for a NBS-LRR protein that also possesses a WRKY domain, though the latter has not yet been shown to be involved in transcription. Interestingly, RRS1 – also known as WRKY52 – is one of the rare exceptions in that it is a resistance protein that interacts directly with its corresponding bacterial avirulence factor PopP2 and both colocalize to the nucleus [56]. If this interaction affects directly the transcriptional activity of defence genes, this would be an extremely condensed defence signalling pathway and it would be interesting to determine what warrants such a pathway as opposed to other signalling pathways that involve multiple layers and nodes. Possible explanations could be that RRS1 activates a single inhibitor of pathogen activity and that this is sufficient for resistance, or that RRS1 activates a "master switch" for the defence response and that this switch activates all other downstream signalling pathways responsible for the cellular changes that are necessary for resistance to occur.

Interestingly, the promoters of pathogen-inducible WRKY genes are significantly enriched in W boxes [44]. It is possible that the auto-regulation of

WRKY genes may provide the positive feedback loop required to amplify the signal of selected resistance genes, as postulated above.

Other transcription factor families involved in the regulation of the defence response include TGA, ERF and MYB factors [57]. The TGA basic leucine zipper (bZIP) DNA-binding proteins were first identified because of their ability to bind tandem repeats of the TGACG sequence in stress-responsive promoters [58]. Some members of the TGA family from *Arabidopsis*, tobacco and rice were later found to bind directly to the NPR1 signalling protein, though not all members bind NPR1 with the same affinity [59-63]. NPR1 does not have a DNA-binding domain itself, but it appears to act as a co-factor that enhances the DNA-binding activity of some TGA factors [60, 64, 65]. This is through a transient TGA-NPR1 interaction that can not be detected in electromobility shift assays and it is therefore not yet known how this interaction stimulates the DNA binding of TGAs.

As mentioned earlier, NPR1 has an important role to play in regulating SA-mediated gene expression. Interestingly, SA stimulates the interaction between NPR1 and the Arabidopsis TGA1 protein [65]. It was found that, upon pathogen recognition, the accumulation of SA in the plant leads to an increase in the cellular reduction potential, and this precedes the accumulation of reactive oxygen species (ROS) and cell death [66, 67]. This increased reduction potential has a two-fold effect. First, it allows the monomerization of NPR1 through the reduction of cysteine residues that form inter-molecular disulfide bonds between NPR1 molecules [67]. These monomers then move from the cytoplasm to the nucleus where they can interact with TGAs [67]. Second, it reduces intra-molecular disulfide bonds in TGA1 (and possibly other TGA factors) that preclude interaction with NPR1 [65]. SA therefore acts indirectly, through changes in the cellular redox potential, to stimulate the DNA-binding activity of TGA factors, via NPR1.

As is the case for WRKY factors, TGA family members can be either activators or repressors of transcription. This is evident in the PR-1 promoter that contains two TGA boxes important for the regulation of this gene. One of them (LS7) acts as a positive cis-element, whereas the other (LS5) acts as a repressor of transcription [68]. Also, silencing of the Arabidopsis *TGA4* and *TGA5* genes led to the activation and repression, respectively, of the octopine synthase promoter in response to pathogen signals, SA and H_2O_2 [69]. This

therefore suggests that TGA4 is a repressor, while TGA5 is an activator of gene expression.

Despite these varied roles, there appears to be some redundancy in the TGA family. It was shown that a single knock-out mutation of *TGA6*, or a double *tga2 tga5* mutation, is not sufficient to block *PR-1* expression in response to defence signals [70]. All three genes are essential however because a triple *tga2 tga5 tga6* mutation completely blocked *PR-1* activation [70].

In contrast to WRKY DNA-binding sites, TGA boxes are not enriched significantly in promoters of genes that are co-regulated in the defence response [71]. This could be because TGA factors can tolerate some variation in the DNA sequence they recognize, and it would therefore not be as straightforward to identify which promoters contain a TGA box [57, 72].

Ethylene-response factors (ERF) are another important class of transcription factors with a role in defence. ERFs bind GCC boxes (GCCGCC) that are usually involved in ethylene responsiveness and, like WRKYs and TGAs, can be either activators or repressors of transcription [73-75]. However, GCC boxes also respond to pathogen signals, including elicitors, JA and SA [76-79]. Interestingly, this response is sometimes independent of ethylene [77]. In fact, SA antagonizes the ethylene-dependent activation of GCC box-containing promoters of defence genes [78].

The genes coding for ERF factors are themselves transcriptionally activated by ethylene, wounding, SA, JA and infection with the bacterial pathogen *P. syringae* [79-83]. Of interest, the tomato gene *JERF1* is activated by JA and ethylene, as well as by salt stress and the plant hormone abscisic acid, suggesting that JERF1 could be a node that integrates biotic, abiotic and developmental signals [83].

The finding that the tomato ERF factors Pti4, Pti5 and Pti6 interacted physically with the protein kinase Pto that confers resistance to bacterial speck disease was the first strong indication that the ERF family of transcription factors is involved in plant defence against pathogens [84]. Pto-mediated resistance to *P. syringae* pv. *tomato* depends on the presence of one of two avirulence proteins (AvrPto and AvrPtoB) in the pathogen, and Pto is therefore considered a R protein [85-88]. While Pto does not possess LRR and NBS domains, as do most R genes characterized to date, it does require

the NBS-LRR protein Prf to confer disease resistance [89]. Pto phosphorylates Pti4, and most likely Pti5 and Pti6 as well [78]. Overexpression of all three *Pti* genes results in enhanced expression of known SA-induced genes, as well as JA and ethylene-induced genes [90]. This was confirmed by microarray and by serial analysis of gene expression (SAGE) with plants overexpressing Pti4. These plants were shown to have induced expression of numerous GCC box-containing genes involved in the defence response [90, 91]. Furthermore, overexpression of *Pti4* or *Pti5* causes enhanced disease resistance [92, 93].

Interestingly, the ERF protein Pti4 also activates genes that do not have a GCC box in their promoter, and it was demonstrated by chromatin immunoprecipitation (ChIP) that Pti4 does bind at least some of these promoters [91]. Pti4 could either bind directly to a non-GCC box site in the DNA, or indirectly by interacting with another transcription factor. To this effect, it is worth noting that, in a recent study, the Pto signalling pathway was found to require two MAPK cascades, as well as the signalling molecule NPR1 and the transcription factors TGA1a and TGA2.2 [94]. As mentioned earlier, expression of NPR1 itself is induced by WRKY transcription factors [52] and there is therefore potential for considerable overlap between the WRKY, TGA, and ERF signalling pathways.

Not surprisingly, the activity of another class of transcription factors involved in disease resistance (MYB) is also closely linked to that of the transcription factors described above.

In contrast to the widely spread R1R2R3 MYB factors, the R2R3 MYB family is unique to plants [95]. R1, R2 and R3 are repeats in the conserved MYB DNA-binding domain. Members of the plant R2R3 MYB family possess two conserved cysteine residues that form a disulfide bridge under non-reducing conditions that prevents DNA binding [96]. This indicates that conditions that favour TGA factor binding (see above) also favour the DNA-binding of plant MYB factors.

As is the case for WRKY factors, the MYB family is very large and not all members involved in the defence response are induced by the same stimuli. For example, tobacco *MYB1* is induced by TMV, incompatible bacterial pathogens and SA [97], whereas the rice *JAMyb* gene is induced by both compatible and incompatible fungal pathogens, as well as by wounding

and JA, but not by SA [98]. It was recently found that both MYB1 and WRK1-3 transcription factors are involved in resistance to TMV conferred by the *N* resistance gene [99]. NPR1 is also involved in this pathway [100], suggesting a link with TGA factors and supporting the role of SA in resistance to TMV and in the activity of MYB1. Interestingly, the same two MAPK pathways (MEK1 and NTF6) involved in Pto signalling with TGA and ERF factors [94, see above] are also required for N-mediated signalling to WRKY and MYB factors [99]. Finally, the promoters of genes regulated by the ERF factor Pti4 were found to be significantly enriched in potential MYB-binding sites [91].

The above list of transcription factors involved in the defence response is by no means exhaustive and other families are emerging in the literature. Nonetheless, these observations highlight the facts that: 1) the transcription factor families described in this chapter do not act independently to activate the defence response, but in a highly coordinated manner, and 2) that there is significant cross-talk between the different signalling pathways that lead to defence gene activation.

Organelles and the defence response

While transcriptional changes in the nucleus contribute to the establishment of disease resistance, other events occur throughout the cell before and after these changes and these rely on a tight coordination of activities in all of the cell's compartments. Such events downstream of incompatible pathogen perception include a rapid and sustained oxidative burst, an activation of calcium signalling and, ultimately, programmed cell death (hypersensitive response – HR) at the site of infection [101]. The primary oxidative burst also causes secondary bursts in distant tissues, leading to cell death in a few, discrete cells and to the development of systemic resistance to a wide range of pathogens (viruses, bacteria, fungi) [102]. The SA signalling pathway is necessary for the establishment of such systemic acquired resistance (SAR) [103].

Disease resistance is compromised when either the oxidative burst or the HR are impaired [104, 105]. In some cases however, cell death does not appear to be absolutely essential for resistance, as evidenced by the identification of mutant plants that are disease resistant while having a much reduced HR [106]. In fact, for some necrotrophic pathogens (that can feed on

dead tissue), cell death actually facilitates infection and is promoted by the pathogen [107].

In animal cells, it is clear that the stress response and programmed cell death (apoptosis) are not solely controlled by transcriptional changes in the nucleus. Organelles such as mitochondria, lysosomes, the endoplasmic reticulum (ER) and the golgi apparatus all participate in stress sensing and are able to initiate signals that will lead either to stress adaptation or cell death [108]. In fact, it appears that dying cells degrade important molecules in all compartments. There is also extensive communication between organelles because mutations in genes with a precise localization can prevent apoptosis induced by damage in another compartment. For example, the endosomal protein RhoB appears to respond to apoptotic signals from the nucleus [109]. Nevertheless, regardless of the origin of the death signal, the final stages of animal apoptosis appear to depend invariably on the "central executioner", which involves caspase activation and/or mitochondrial membrane permeabilization and release of proteins such as cytochrome c from the mitochondria [108].

Obvious sequence homology with animal caspases has not been found in plants, though caspase activity has been detected and animal caspase inhibitors inhibit programmed cell death in plant cells [110, 111]. In addition, caspase activity has also been associated with the plant HR and disease resistance [110]. Plant vacuolar processing enzymes (VPE) display structural homology to animal caspases [112]. They were found to have caspase activity and to be responsible for programmed cell death induced by tobacco mosaic virus in tobacco and by the bacterial pathogen *P. syringae* pv. tomato DC3000 in *Arabidopsis* [113, 114]. Another class of proteases common to fungi and plants, the metacaspases, display weak sequence homology to animal caspases, do not have caspase activity in vitro, but induce caspase activity and cell death in vivo [112]. Metacaspases are induced by both compatible and incompatible pathogens, suggesting their involvement in the defence response [112, 115, 116]. This is in contrast to animal caspases that tend to be constitutively expressed in an inactive form and activated by processing. Another interesting difference between plant and animal caspases is intra-cellular localization. While animal caspases are cytosolic, except for a few exceptions such as the ER caspase-12 [117] and

the nuclear caspase-2 [118], plant VPE are localized to the vacuoles [119] and some metacaspases are predicted to be mitochondrial and chloroplastic [112].

Mitochondrial membrane permeabilization [120] and cytochrome c relase [121] also occur in plants at the onset of cell death, and it has been found that mitochondria play a role in the HR [122]. It therefore appears that signals that trigger the HR also converge on an "executioner", though it is probably not as "central" as that seen in animal cells, because of the varied localization patterns of plant caspase-like proteins.

Important redox changes are also a constant in plant and animal stress adaptation and programmed cell death, though there are once again significant differences. Whereas the oxidative burst seen in animal phagocytes originates mainly from a NADPH oxidase present at the plasma membrane and at the surface of phagosomes [123], there are a multitude of sources of reactive oxygen species (ROS) in elicited plant cells. These sources are both intra- and extracellular and include cell wall peroxidases and amine oxidases, plasma membrane-bound NADPH oxidases and intracellular oxidases and peroxidases in mitochondria, chloroplasts, peroxisomes and nuclei [104, 124-127]. It is not clear to what extent each system contributes to the oxidative burst, but it is likely that they act in concert. It was found for example that even a slight amount of oxidative stress could elicit H_2O_2 production by the mitochondrial respiratory chain (complexes I and III) of non-photosynthetic tobacco cells and lead to membrane permeabilization and cell death [128]. Multiple sites of ROS synthesis therefore allow signal amplification, while providing the cell with many control checkpoints. Moreover, the fact that synthesis of ROS can be accomplished in various organelles allows the compartmentalization of downstream responses.

Ozone elicits responses very similar to those caused by biotic elicitors in plants, including the SAR [129]. In a recent study on the effect of ozone in plant epidermal cells, it was found that the first site of ROS production (within 5 minutes of ozone treatment) was the chloroplasts of guard cells [130]. Subsequently, the membrane NADPH oxidase of these guard cells became activated, produced diffusible H_2O_2 and this, in turn, induced ROS production in various compartments of neighbouring cells [130]. It therefore appears that,

at least under these conditions, chloroplasts play a central role in initial stress signalling.

There is considerable evidence linking chloroplasts with disease resistance. For example, altering porphyrin metabolism in chloroplasts induced defence genes and conferred increased resistance to TMV [131]. A recessive mutation in the plastid fatty acid desaturase SSI2 [132] also induces SA accumulation, PR gene activation and disease resistance in a NPR1-independent manner [133]. Interestingly, *ssi2* mutants have impaired JA-dependent gene activation, suggesting that SSI2 mediates cross-talk between the JA and SA pathways of defence signalling [132], as does the transcription factor WRKY70 mentionned above. Overexpression of a truncated version of SSI2, missing the putative N-terminal chloroplast transit peptide, is unable to rescue the *ssi2* mutation [134]. Furthermore, the defects observed in *ssi2* appear to be caused by a reduction in soluble 18:1 chloroplast fatty acids in these plants, in relations to the levels of 18:0 [134]. The *act1* mutation, which causes an increase in 18:1 fatty acid levels in the chloroplast is able to completely reverse the effects of *ssi2* [134]. These results strongly suggest that the site for JA and SA cross-talk is the chloroplast and that it involves fatty acid signalling.

A portion of the cellular SA is synthesized in the chloroplast and this portion is necessary for disease resistance [135]. Also, at least one SA-binding protein (SABP3) is chloroplastic [136]. Interestingly, SA also binds and inhibits catalase [137, 138], an enzyme present in the peroxisomes that detoxifies ROS produced following photorespiration in the chloroplast and is important for stress defence [139]. Photorespiration is a wasteful process that serves as an energy dissipation mechanism to avoid overproduction of ROS in conditions of stress, excess light and/or low carbon fixation [140]. Under such conditions of excess excitation energy (EEE) [141], the photosynthetic electron transport chain becomes over-reduced, and this gives rise to the production of ROS and to a phenomenon known as photoinhibition, which is the degradation of photosystem II following oxidative damage. If the oxidative stress exceeds the inherent detoxification capacities of the cell, this ultimately leads to cell death. Photorespiration is one of a number of energy dissipation mechanisms under these conditions.

Further evidence for the role of photorespiration in pathogen defence comes from a recent study that showed that SHMT1 is a serine hydroxymethyltransferase that functions in the chloroplast photorespiratory pathway and provides resistance to both biotic and abiotic stresses [142]. Mutations in this gene caused excess production of H_2O_2 and resulted in the appearance and uncontrollable spread of leaf lesions following abiotic stress and pathogen infection [142]. Mutations in the *lesion simulating disease 1* (*LSD1*) gene cause a similar phenotype and were recently found to be associated with a deficiency in the dissipation of EEE by photorespiration [143]. The LSD1 protein is a novel zinc finger protein [144] and it has been proposed that it controls catalase during acclimation to EEE [143].

Summary

While the gene-for-gene theory dates from the 1940s, the first plant resistance gene was cloned in 1993 [145], less than 15 years ago. Since then, our understanding of plant disease resistance has progressed dramatically. In 2000, the first complete genome sequence of a higher plant, *Arabidopsis*, was published [146]. This achievement, combined with the advent of tools for whole genome analysis (genomics) has allowed the study of multiple co-regulated genes and has highlighted the fact that disease resistance stems from the interaction of numerous pathways. It is very logical that it would be so. To produce defence compounds, carbon needs to be diverted from primary metabolism. This carbon is fixed by photosynthesis in tissues known as source and distributed to sink tissues. Regulation of photosynthesis and the movement of photosynthates are therefore likely to be intimately linked to disease resistance. Cell death is triggered at the site of infection but needs to be controlled in the surrounding cells. ROS play a role in both these situations. Furthermore, ROS act as signalling molecules but are toxic. They need to be synthesized and degraded, depending on the cellular context. All these actions call upon multiple enzymes and biochemical pathways in numerous cellular compartments. Most importantly, all these actions need to be tightly coordinated.

The future of plant disease resistance research lies in the understanding of this coordination and how it is achieved. This thesis aims to be part of this effort.

Objectives

The primary objective of this thesis was to clone and characterize the gene coding for the DNA-binding protein in the plant transcription factor PBF-2, responsible for the elicitor-dependent activation of the defence gene *PR-10a*. Specific objectives were:

- To screen a complementary DNA (cDNA) library for sequences corresponding to two small peptides sequenced from the PBF-2 factor;
- To clone the full-length gene;
- To verify that the protein encoded by this gene is part of PBF-2;
- To purify the recombinant protein to produce an antibody;
- To verify the intra-cellular localization of this protein using a green fluorescent protein (GFP) fusion in both transient and stable expression;
- To produce and characterize transgenic plants over- and underexpressing this gene;

This project was intended to improve the knowledge of transcriptional activation during the defence response. Unexpected findings allowed us to show a role for the DNA-binding protein of PBF-2 in the coordination of stress-dependent gene expression in nuclei and chloroplasts.

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Chapter II

Cloning of a novel transcriptional activator

Introduction

Phytophthora infestans, the causal agent of the potato late blight disease, is mostly known as one of the factors involved in the Irish Great Potato Famine of 1837. Incompatible races of this oomycete, however, cause typical hypersensitive response symptoms on potato leaves and tubers [1]. A cell-free homogenate of *P. infestans* is also able to elicit the hypersensitive response in tubers. The molecules with elicitor activity in such extracts were found to be the twenty-carbon polyunsaturated fatty acid, arachidonic acid and eicosapentaenoic acid, and both are able to cause the hypersensitive response on their own [2].

Gene expression during the potato hypersensitive response was investigated using in vitro translation of mRNAs isolated from tubers elicited with arachidonic acid. The accumulation of at least 16 mRNAs varied, of which 13 were more expressed following elicitation [1]. One of these induced genes was found to be *PR-10a* (formerly known as *STH-2*). *PR-10a* is induced in tubers either wounded, elicited with arachidonic or eicosapentaenoic acid, or treated with *P. infestans* homogenate [1, 3, 4, 5]. Live compatible and incompatible strains of *P. infestans* also elicit this gene, but compatible strains show a smaller induction [4]. In leaf tissues, however, only the homogenate [3] and live pathogen [4] are able to induce *PR-10a* gene expression. In tubers, the induction is rapid (detectable 8 hrs after treatment), reaches a maximum at 24 hrs, and is sustained well after 72 hrs post-elicitation [3]. It was found that anaerobiosis can inhibit the accumulation of the *PR-10a* mRNA in elicited tuber discs [3]. A study involving a fusion of the *PR-10a* promoter with the β-glucuronidase (GUS) gene allowed a more extensive study of *PR-10a* gene expression patterns. Histochemical staining of elicited or infected tissues showed that *PR-10a* induction was strongest in vascular bundles [5]. As defence signals are known to be transported through the vasculature [6, 7] to distal portions of the plant, it is possible that PR-10a could be involved in the synthesis of such a signal. Interestingly, the *PAL* gene, involved in the synthesis of SA, as well as the *PR-1* gene, are also

expressed in vascular bundles [8, 9]. Alternatively, PR-10a could be involved in assimilate metabolism or movement, as the defence response is often characterized by a source-to-sink transition involving mobilization of sugar reserves through the vasculature [10, 11]. In healthy tissue, GUS staining could only be seen at the surface of the stigma [5]. This finding is intriguing given the fact that *PR-10* genes are homologous to the major allergen of birch pollen [12], though GUS staining could not be detected in pollen. Given the fact that both the defence response and stigma-pollen interactions involve self/non-self recognition, it is possible that these processes use similar schemes. This would explain the apparent dual role of *PR-10a* suggested by its localization in two different cell types.

To gain insight into the regulation of *PR-10a* transcription during the defence response, promoter deletion analysis was undertaken [13]. It was found that a 30 bp region between -135 and -105 was necessary and sufficient for the elicitor responsiveness of the PR-10a promoter. This region was therefore called the Elicitor Response Element (ERE). Strong activation of the promoter, however, required the presence of an enhancer region between -155 and -135. Finally, a negative regulatory region was found between -52 and -27. Interestingly, in transient expression assays in potato mesophyll protoplasts, the ERE was able to activate transcription, even in the reverse orientation, and duplication of this element led to almost twice the levels of transcription observed with a single ERE element [13].

The ERE was specifically recognized in vitro by a nuclear factor, PBF-2 (for *PR-10a* binding factor 2), isolated from potato tubers [14]. Binding of PBF-2 to the ERE after wounding or elicitation with arachidonic acid correlated with the accumulation of *PR-10a* mRNA [14]. Furthermore, both *PR-10a* gene expression and PBF-2 binding to the ERE are controlled by a functional homologue of protein kinase C (PKC) [15]. These results suggest that PBF-2 could play an important role in the activation of *PR-10a* during the defence response.

To further characterize the function of PBF-2, it became important to isolate its constituent protein(s). Interestingly, PBF-2 was found to bind single-stranded DNA, as it could bind with high affinity to both the coding (CS) and the non-coding strand (NCS) of the ERE [16]. It was therefore purified, from potato tubers elicited with arachidonic acid, by a combination of anion

exchange chromatography and two rounds of DNA-affinity chromatography using a biotinylated form of the NCS. After the first round of DNA-affinity chromatography, three proteins of 105, 48, and 24 kD could be detected but, after the second round, only the 24 kD protein was found [16]. PBF-2 DNA-binding activity could not be detected in crude nuclear extracts of fresh tubers. However, a comparable amount of DNA-binding activity as with elicited tubers could be detected when the fresh tuber extracts were purified by anion-exchange chromatography [16]. This suggests that PBF-2 is present in an inactive form in fresh tuber nuclei, and that its DNA-binding activity is activated upon elicitation.

Purified PBF-2 was UV cross-linked to radio-labelled NCS, digested with DNase I, and electrophoresed on a SDS-polyacrylamide gel. A single 24 kD protein could be detected in this way, suggesting that it is the DNA-binding component of PBF-2. It is hereafter designated as p24. In order to clone the gene encoding p24, the purified protein was excised from a polyacrylamide gel and digested with trypsin. After capillary electrophoresis, two peptides were selected and sequenced by Edman degradation. The partial amino acid sequences of the two peptides obtained in this way were: SPEFSPLDSGAFK and VEPLPDG.

This chapter presents the cloning of p24, a novel single-stranded DNA-binding protein. Recombinant p24 showed the same DNA-binding sequence specificity as the purified PBF-2 factor and was shown to be the DNA-binding component of PBF-2 [16].

Materials and methods

Cloning of p24

A partial tomato expressed sequenced tag (EST) sequence (AI488224.1) coding for the p24 large peptide (SPEFSPLDSGAFK) and an Arabidopsis bacterial artificial chromosome (BAC) clone (AC002521) coding for both peptides were aligned and polymerase chain reaction (PCR) primers were designed to flank the large peptide. The primers derived from the tomato EST sequence were as follows: 5'-ATATACAAAGGGAAGGCAGT and 5'-GATAGATCCAATTTCAGTCAC. These primers were first used to amplify potato genomic DNA. A single DNA fragment of ~550 bp was amplified,

cloned, partially sequenced, and shown to share 99% identity over 109 bp with the tomato sequence and to code for the large peptide (data not shown).

As illustrated in Figure 2.1, a modified version of the method described by Israel [17] was used to screen a potato cDNA library made in Lambda ZAP (Stratagene) from mRNAs isolated from potato tubers elicited with arachidonic acid for 72 hr [3]. Approximately 960 000 plaque-forming units (pfu) were used to infect *Escherichia coli* XL-1 Blue (Stratagene), and aliquots were dispensed in a 96-well microtiter plate at 10 000 pfu per well in 100 µL. The phages were amplified for 8 hrs at 37°C. An aliquot from each well was mixed with an equal volume of water, and 3 µL from each sample was used in a PCR reaction with the *p24* primers described above. Approximately 336 000 pfu from a positive well were aliquoted in another 96-well plate at 4 000 pfu per well, amplified, and analyzed by PCR as above. This process was repeated a total of four times, with 3 000 and 250 pfu per well in the third and fourth screens, respectively. Isolation of the *p24* cDNA clone was done by hybridization of plaques from a positive well with the genomic DNA fragment described above. A positive clone was excised using the ExAssist system (Stratagene), according to manufacturer's instructions. The clone was sequenced on both strands.

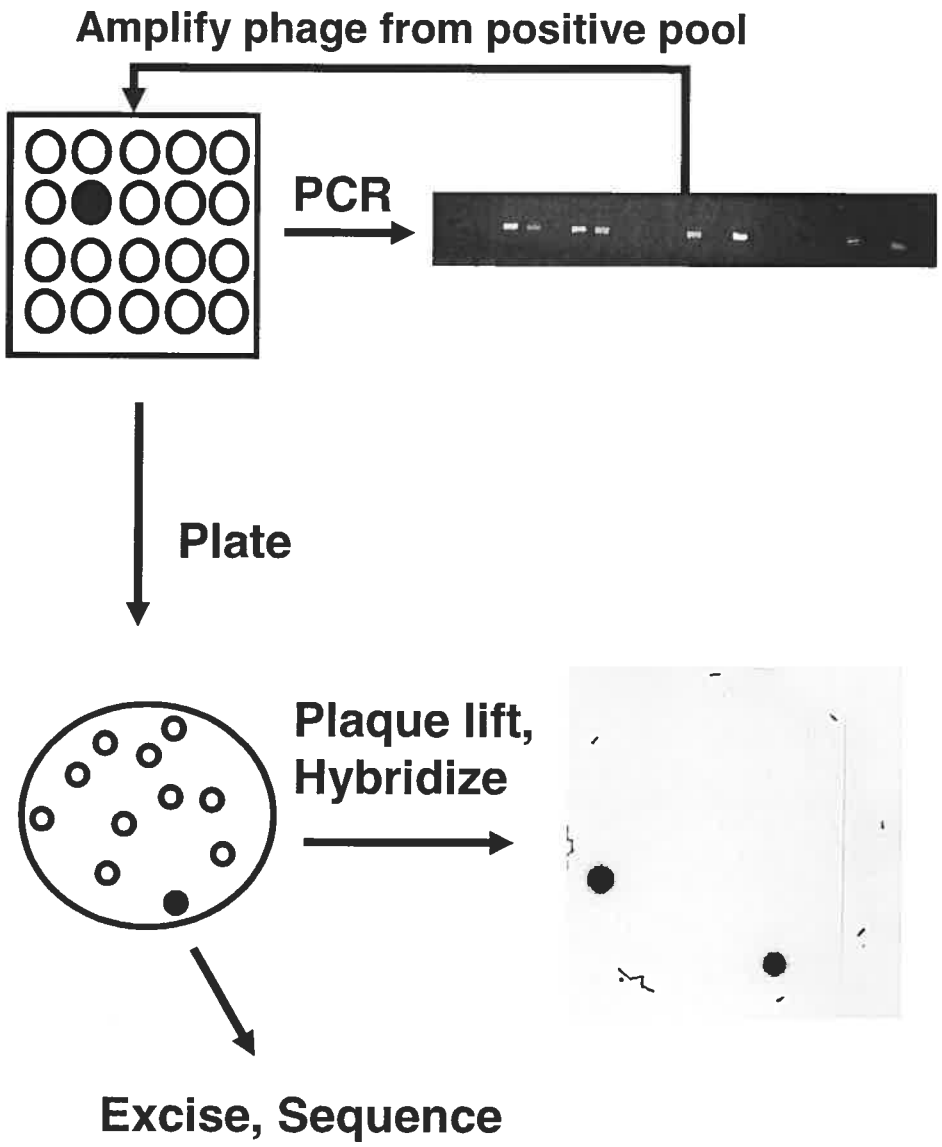
DNA gel blot analysis

Genomic DNA from wild-type potato (cultivar Kenebec) was extracted using 3% (w/v) hexadecyltrimethylammonium bromide (CTAB), as described previously [18]. The DNA (10 µg) was digested for 4 hours with the restriction enzymes *Eco*RI, *Bam*HI, *Hind*III and *Hae*III. The DNA fragments were then separated overnight in a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was baked at 80°C for 2 hours and stored at room temperature until it was used for hybridization.

The *p24* cDNA clone was random-primer labelled with [α^{32}P]dCTP. The probe was then purified on a home-made 1 mL Sephadex G-50 (Pharmacia) column. Membrane hybridization was done as described [19].

Figure 2.1

Rapid PCR screening of a cDNA library. A cDNA library in phages is separated into 96 pools. Each pool is tested by PCR for the presence of a gene of interest. A positive pool is identified, the phage is amplified, and the new library is further separated into 96 pools. This process is repeated 4 times. The phages from the final positive pool are plated onto Petri dishes, transferred to membranes and hybridized with a gene-specific probe. Phages from a positive plaque are excised and the insert is sequenced.


Amplify phage from positive pool
PCR
Plate
Plaque lift,
Hybridize
Excise, Sequence

Expression and purification of the recombinant protein

The *p24* coding region was amplified by using PCR primers (5'-CCAAAAATCTCTTGGATCCATGTCC and 5'-CCAGAACTCGAGATTCCAT-TC) that inserted a *Bam*H1 site immediately preceding the ATG and a *Xho*1 site after the STOP codon, respectively. The PCR product was purified and inserted into the *Bam*H1 and *Xho*1 sites of the pET-21a vector (Novagen), creating a fusion protein with a T7 tag at the N-terminus and a histidine tag at the C-terminus. The truncated version of the p24 protein was produced by using the same *Xho*1 primer and a primer (5'-TTAACATGTCGCGGATC-CGATTATTTTG) inserting a *Bam*H1 site 67 amino acids from the N-terminus. These constructs were made in XL-1 blue *E. coli* cells (Stratagene) and then transferred into the expression strain BL21 pLysS (Novagen). A single colony from the latter was then grown at 37°C, and protein expression was induced for 3 hrs using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested and resuspended in START buffer (20 mM Na_2HPO_4 pH 7.2, 500 mM NaCl). The cells were lysed by freeze-thaw and sonication, then centrifuged at 11 000 g for 1 hr at 4°C. One volume of 50% (w/v) PEG 8000 was added to the supernatant and the precipitated proteins were centrifuged at 11 000 g for 1 hr at 4°C. Pellets were washed and resuspended in START buffer. Fusion proteins were purified using HiTrap affinity columns and a fast protein liquid chromatography (FPLC) apparatus (Amersham Parmacia Biotech), according to manufacturer's instructions. Purified proteins were eluted in START buffer containing 50 mM EDTA and were stored a -80°C in 10% (v/v) glycerol. For EMSA, the eluted samples were first diluted 1:1 in EMSA buffer (20 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA).

Electrophoretic mobility shift assays

Single-stranded synthetic oligonucleotides for non-coding strand of the -130 to -105 region of the ERE (5'-CTAGACCATTTTTGACATTTGTGTCAT-TTTATCTAG) were labelled using T4 polynucleotide kinase [16]. Reaction mixtures contained 1 μL (20 000 cpm) of end-labelled nucleotide and 40 μL of purified protein with a final EDTA concentration of 50 mM. Reactions were performed at room temperature for 15 min and subsequently loaded on a 5.4% polyacrylamide gel (29:1 acrylamide:bisacrylamide in 100 mM Tris-HCl pH 8.0, 100 mM borate, 2 mM EDTA). After electrophoresis, the gels were

blotted onto Whatman 3MM paper and autoradiographed at -80°C on Kodak XAR film.

Gel filtration chromatography

The PBF-2 complex was purified as described, using anion-exchange chromatography, from potato tubers elicited for 9 hours with arachidonic acid [16]. The purified proteins were loaded onto a Superose 12 (Pharmacia) gel filtration chromatography column in EMSA buffer containing 200 mM NaCl. Fractions of 0.5 mL were collected and subjected to EMSA using the non-coding strand of the ERE as a probe (see above).

Results and discussion

Cloning of p24

The PBF-2 factor was previously purified from elicited tubers [16] and peptide sequencing from the p24 protein revealed two peptides (SPEFSPLDSGAFK and VEPLPDG) that showed no significant similarity to proteins of known function. The large peptide sequence, however, was encoded by a partial expressed sequenced tag (EST) from tomato carpel tissue extracted 5 days pre-anthesis to 5 days post-anthesis (accession AI488224.1). This EST showed homology to an *Arabidopsis thaliana* BAC clone (accession AC0022521) from chromosome II that also encoded the other peptide sequence of p24. The sequence of a fragment amplified from potato genomic DNA encoded the large peptide and aligned with the tomato EST and the Arabidopsis BAC clone (data not shown). The same PCR primers were used to amplify a single fragment from a potato cDNA library constructed from tubers elicited with arachidonic acid for 72 hours. Using a PCR-based cDNA pooling approach [17], we isolated a cDNA clone for *p24*. The frequency of this cDNA clone in the 72-hr elicited cDNA library was found to be approximately 0.0000083 (1/120 000), suggesting that the *p24* gene is not highly expressed after long periods of elicitation.

The cDNA clone revealed a single open reading frame encoding a protein of 274 amino acids. As indicated in Figure 2.2, both peptides from the purified p24 were present in the encoded protein. This protein has a predicted molecular weight of 30.3 kD, suggesting that the protein may be processed.

Figure 2.2

Amino acid sequence encoded by the *p24* gene. The two peptides obtained by sequencing of protein fragments from the purified PBF-2 factor are shown in red. The poly-Q domain is shown in blue. The putative peptide processed from the mature p24 protein is underlined.

```
  1 MSNFSLSPSPTSGFSLNLQN
 21 PTKTSYLSFSSSINTIFAPL
 41 SSNTTKSFSGLTHKAALPRN
 61 LSLTCRHSDYFEPQQQQQQQ
 81 QQQPQGASTPKVFVGYSIYK
101 GKAALTVEPRSPEFSPLDSG
121 AFKLSREGMVMLQFAPAAGV
141 RQYDWSRKQVFSLSVTEIGS
161 IISLGAKDSCEFFHDPNKGR
181 SDEGRVRKVLKVEPLPDGSG
201 HFFNLSVQNKLINLDENIYI
221 PVTKAEFAVLVSAFNFVMPY
241 LLGWHTAVNSFKPEDASRSN
261 NANPRSGAELEWNR
```

p24 is part of a small gene family

The *p24* gene is unique to plants and ESTs encoding proteins with strong similarity to p24 can be found from evolutionarily distant plants such as loblolly pine, rice, maize, Arabidopsis and tomato (Figure 2.3). This conservation of sequence suggests that p24 may play an important role in fundamental processes in the plant. Figure 2.4 shows a phylogenetic tree of the plant p24 sequences.

A DNA gel blot of potato genomic DNA hybridized with a *p24* probe suggests that there are three to five members of this gene family in potato (Figure 2.5). It is also possible that some of the restriction site patterns observed are partly due to different alleles, as potato plants are tetraploid.

PBF-2 is a 100 kD complex

Gel filtration chromatography of PBF-2 purified from nuclei of elicited potato tubers showed that PBF-2 is a complex of at least 100 kD (Figure 2.6). Two other proteins, of 48 and 105 kD, co-purify with p24 up to the first round of DNA-affinity chromatography [16] and it is possible that they are part of the PBF-2 complex. The crystal structure of p24, however, has since revealed that p24 is found as a tetramer [20], which explains the molecular weight of ~100 kD. It is therefore likely that, in elicited tissues at least, PBF-2 consists only of p24. In fresh tissues on the other hand, the presence of an inhibitor in the PBF-2 complex cannot be ruled out, as it has been shown that anion-exchange chromatography is necessary to uncover the DNA-binding activity of PBF-2 [16]. It will be interesting to perform gel filtration chromatography on fresh potato tuber extracts, to see if PBF-2 elutes at a higher molecular weight.

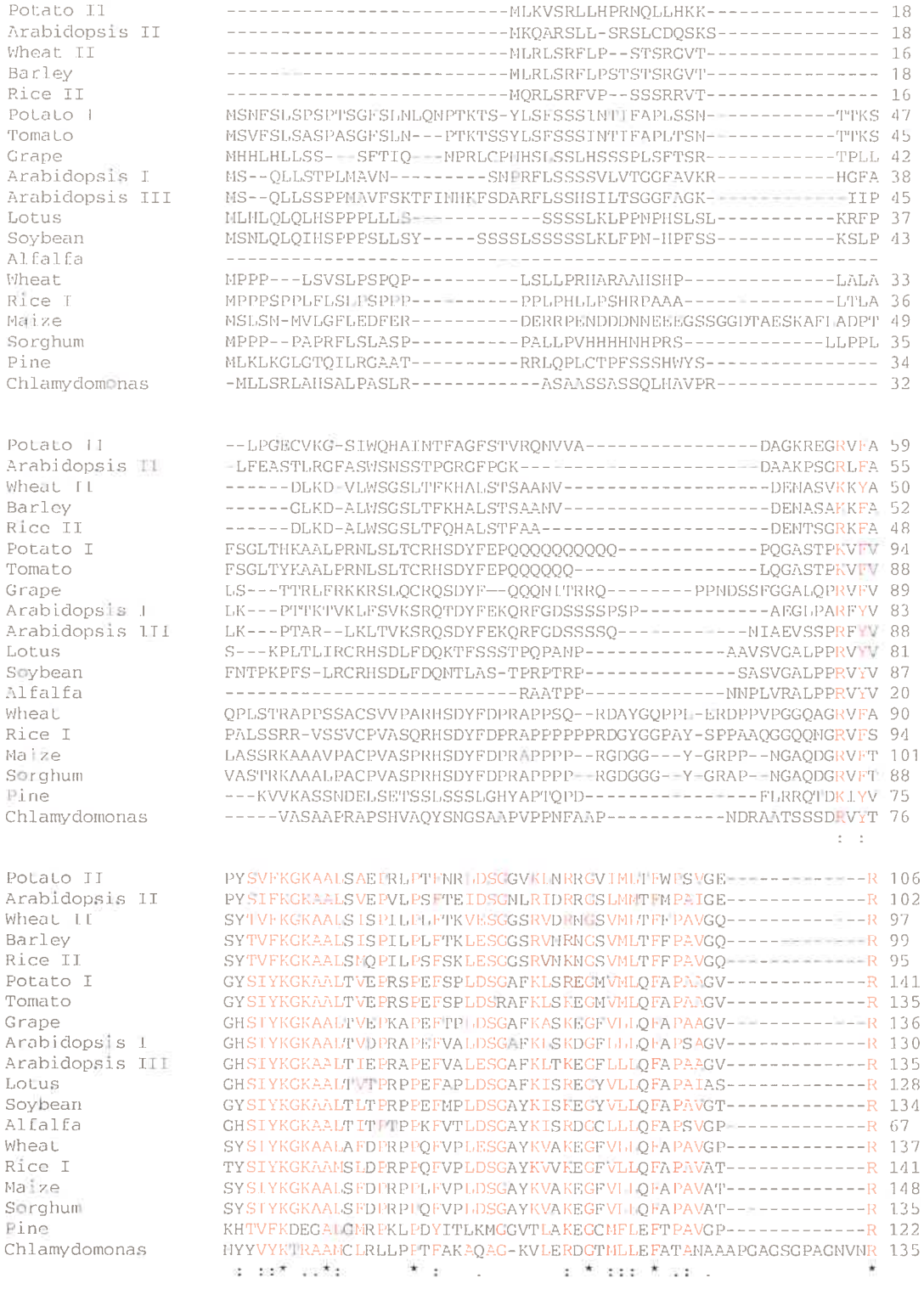
p24 possesses a glutamine-rich domain

Strikingly, a stretch of 11 glutamine residues interrupted by only one proline can be found in the N-terminal half of p24. Such glutamine stretches are part of the proline/glutamine class of transcriptional activation domains. Polyglutamine stretches have been found to activate transcription in human HeLa cells [21] and in plant cells [22]. Interestingly, all *p24* sequences found in the GenBank database fall into two main groups (Figures 2.3 and 2.4) that differ mainly in the presence/absence of this polyglutamine domain. This

Figure 2.3

The p24 protein sequence is evolutionarily conserved. Proteins with similarity to the full-length potato p24 sequence were extracted from the GenBank database and aligned with the Clustal W multiple sequence alignment program (http://www.ebi.ac.uk/clustalw/), using the default settings. Protein sequences used were from the following accession numbers: potato I (AAF91282), Arabidopsis I (AAG48815), Arabidopsis II (AAL59932), Arabidopsis III (AAU15140), rice I (BAD68773), and rice II (BAD28177). In all other cases, protein sequences were translated from nucleotide expressed sequence tag (EST) sequences. For some, more than one EST had to be assembled to form a complete protein sequence. Accession numbers were as follows: potato II (BQ506067, BQ506068), Chlamydomonas (B1717574, BU650445, BU649234), wheat I (CD373469, BE426410, CA498265), wheat II (CD871144, CJ538235), tomato (AW222339, AI488224), grape (CB339732), barley (BF627441), lotus (CN825759, AV765571), maize (CF007396, CD650748), alfalfa (BE202518, AL373682), pine (DT635552), sorghum (CN148887), and soybean (BU547135, CA782570). All sequences, except for the alfalfa sequence, are presumed to be complete protein sequences. Conserved amino acids are shown in red.

Symbols: * residues are identical in all sequences in the alignment
: conserved substitutions are observed
. semi-conserved substitutions are observed


Potato II
Arabidopsis II
Wheat II
Barley
Rice II
Potato I
Tomato
Grape
Arabidopsis I
Arabidopsis III
Lotus
Soybean
Alfalfa
Wheat
Rice I
Maize
Sorghum
Pine
Chlamydomonas

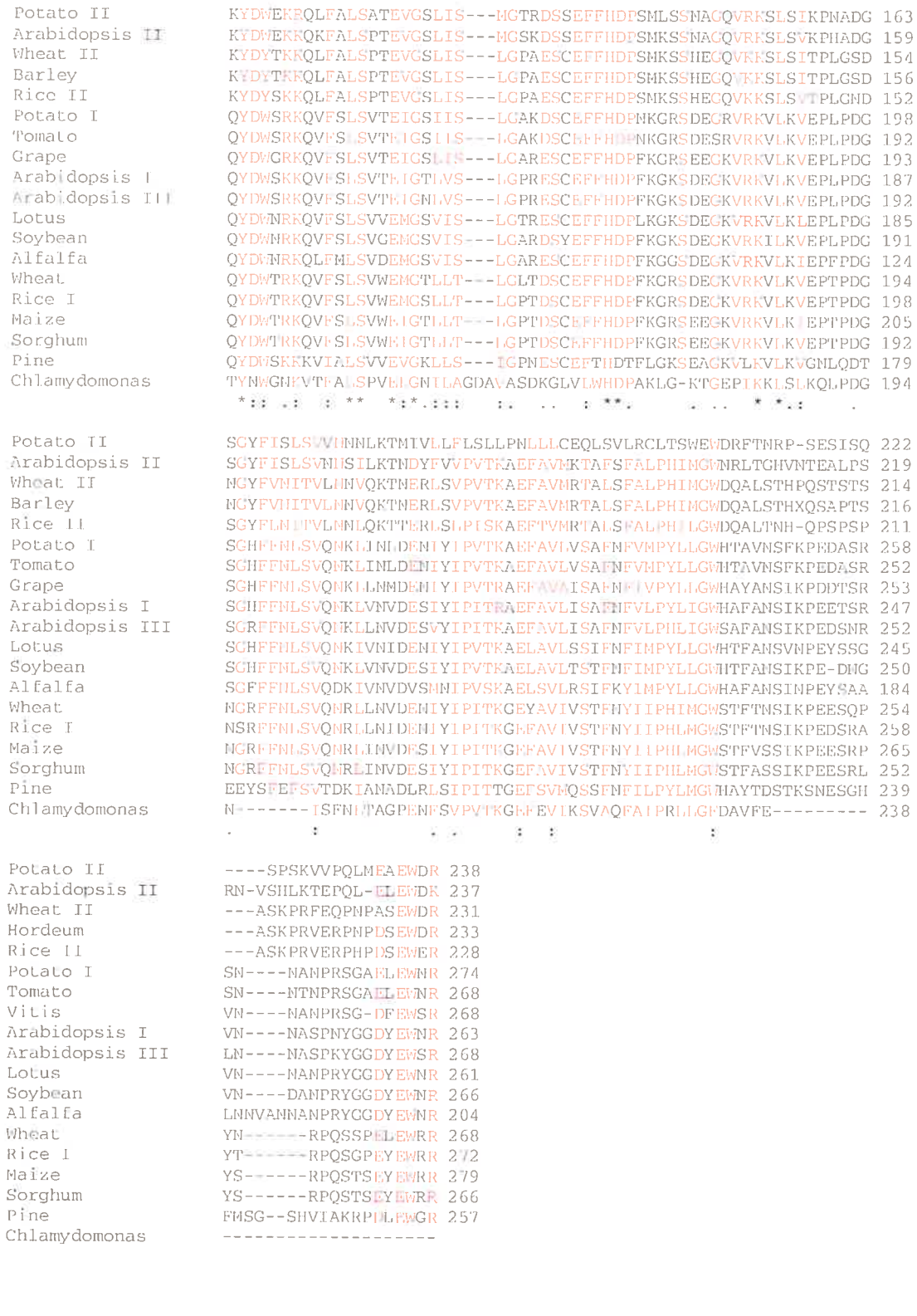

Potato II
Arabidopsis II
Wheat II
Barley
Rice II
Potato I
Tomato
Grape
Arabidopsis I
Arabidopsis III
Lotus
Soybean
Alfalfa
Wheat
Rice I
Maize
Sorghum
Pine
Chlamydomonas
Potato II
Arabidopsis II
Wheat II
Barley
Rice II
Potato I
Tomato
Grape
Arabidopsis I
Arabidopsis III
Lotus
Soybean
Alfalfa
Wheat
Rice I
Maize
Sorghum
Pine
Chlamydomonas
Potato II
Arabidopsis II
Wheat II
Hordeum
Rice II
Potato I
Tomato
Vitis
Arabidopsis I
Arabidopsis III
Lotus
Soybean
Alfalfa
Wheat
Rice I
Maize
Sorghum
Pine
Chlamydomonas

Figure 2.4

There are two major groups of p24 sequences. Phylogenetic tree of the sequences shown in Figure 2.3. The tree was obtained using the Multalin program (http://prodes.toulouse.inra.fr/multalin/multalin.html). Default settings were used. PAM = Point Accepted Mutations per 100 aligned positions.

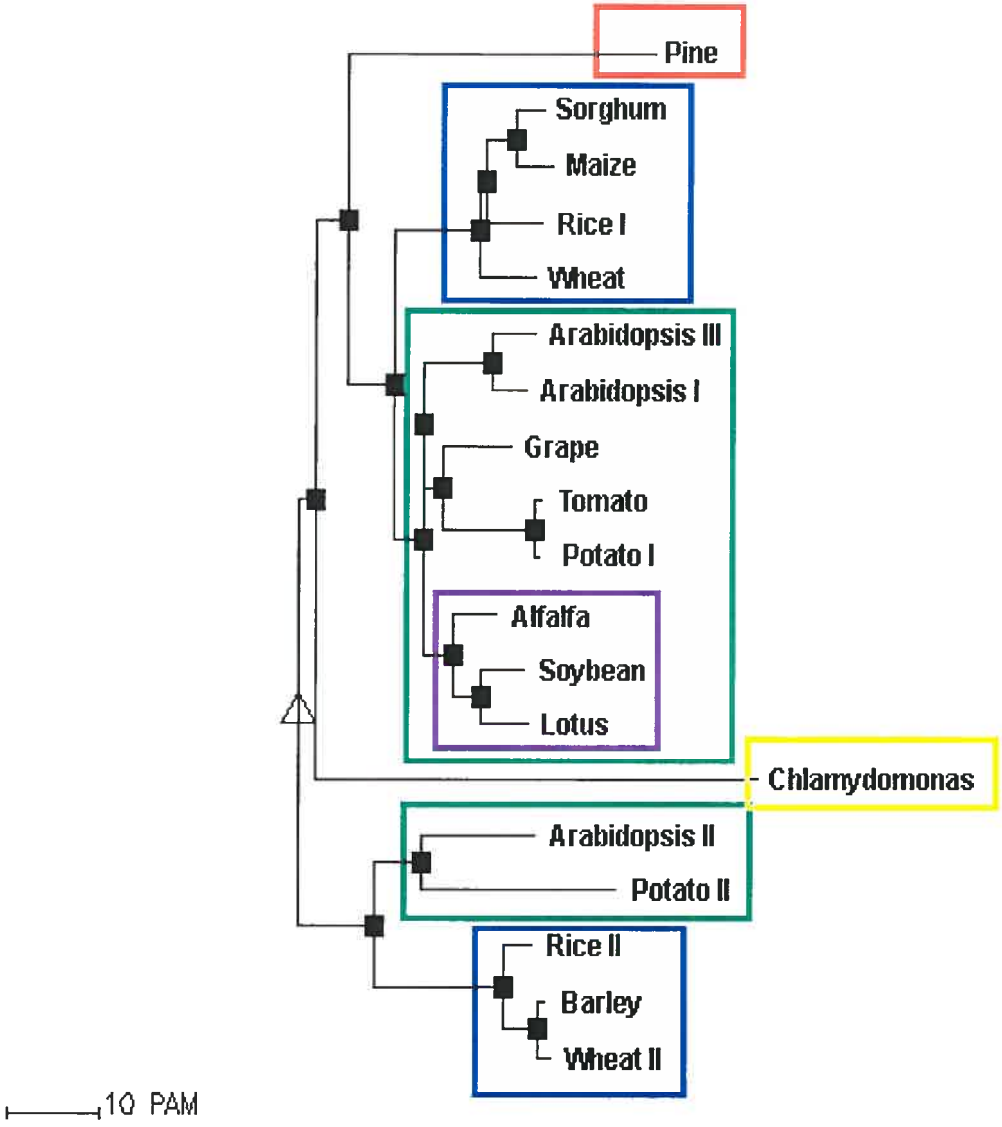

Pine
Sorghum
Maize
Rice I
Wheat
Arabidopsis III
Arabidopsis I
Grape
Tomato
Potato I
Alfalfa
Soybean
Lotus
Chlamydomonas
Arabidopsis II
Potato II
Rice II
Barley
Wheat II
10 PAM
Gymnosperms
Monocotyledons
Dicotyledons
Nitrogen-fixing plants
Algae

Figure 2.5

p24 belongs to a small gene family in potato. DNA gel blot of potato genomic DNA digested with 4 restriction enzymes and probed with the p24 cDNA.

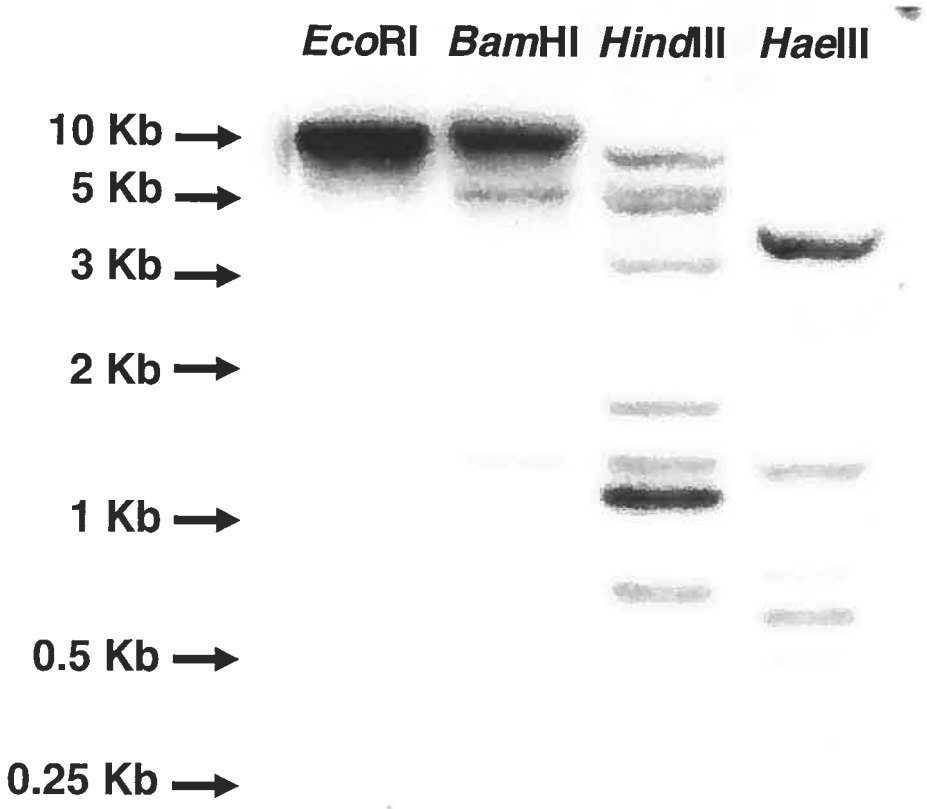
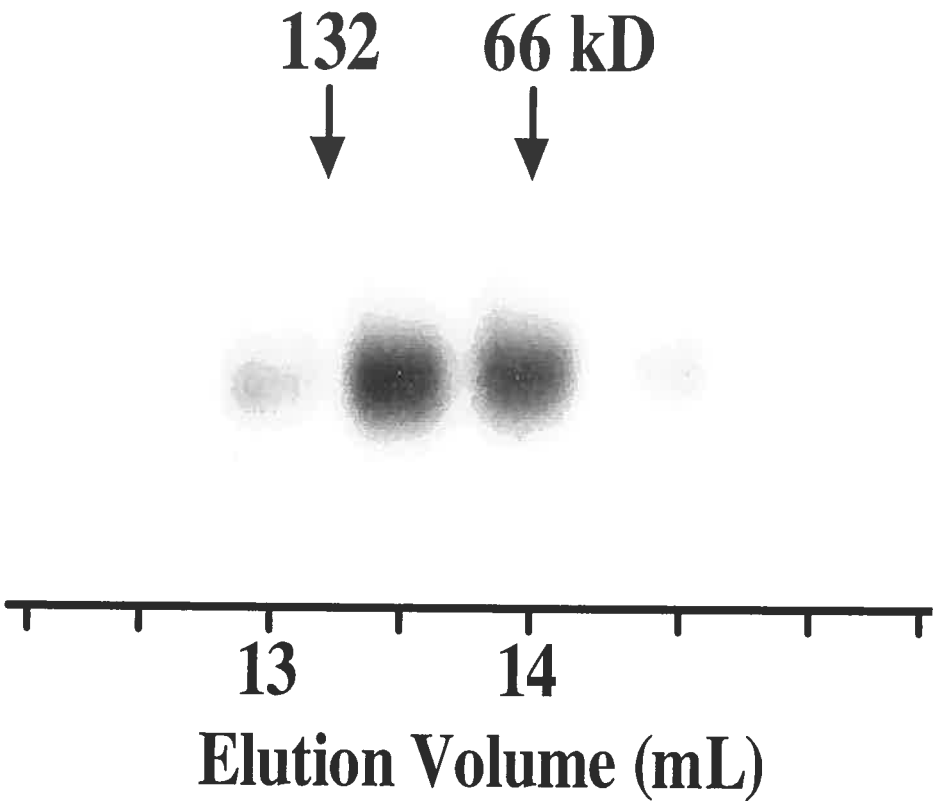

*Eco*RI
*Bam*HI
*Hind*III
*Hae*III
10 Kb
5 Kb
3 Kb
2 Kb
1 Kb
0.5 Kb
0.25 Kb

Figure 2.6

PBF-2 is a protein complex of approximately 100 kD. Gel filtration chromatography of the PBF-2 complex in elicited potato tubers. Each 500 µL elution fraction was subjected to EMSA. The elution volume is indicated. Protein sizes correspond to protein standards eluted in parallel, under the same conditions.


132
66 kD
13
14
Elution Volume (mL)

raises the possibility that not all members of the p24 gene family act as transcriptional activators, or that they do not all act in the same way.

Glutamine-rich activation domains are known to activate transcription preferentially from proximal promoters, in concert with enhancer elements [23]. Similarly, PBF-2 acts from a proximal element (-135 to -105) of the PR-10a promoter, and distal enhancer elements (-155 to -135 and -670 to -441) are required for high expression of *PR-10a* [14]. In plant cells, a fusion protein containing the GAL4 DNA binding domain, the VP16 activation domain, and a stretch of 51 glutamine residues activated transcription 14-fold more than did the GAL4/VP16 fusion alone [22]. Studies in animal cells have shown that fusion proteins with a tract of 10 glutamines displayed the most transcription, while proteins with >26 glutamine residues showed progressively less transcription [21]. These observations suggest that the polyglutamine domain found in p24 has the potential to activate transcription. This has since been shown, using transient expression in plant cells [24].

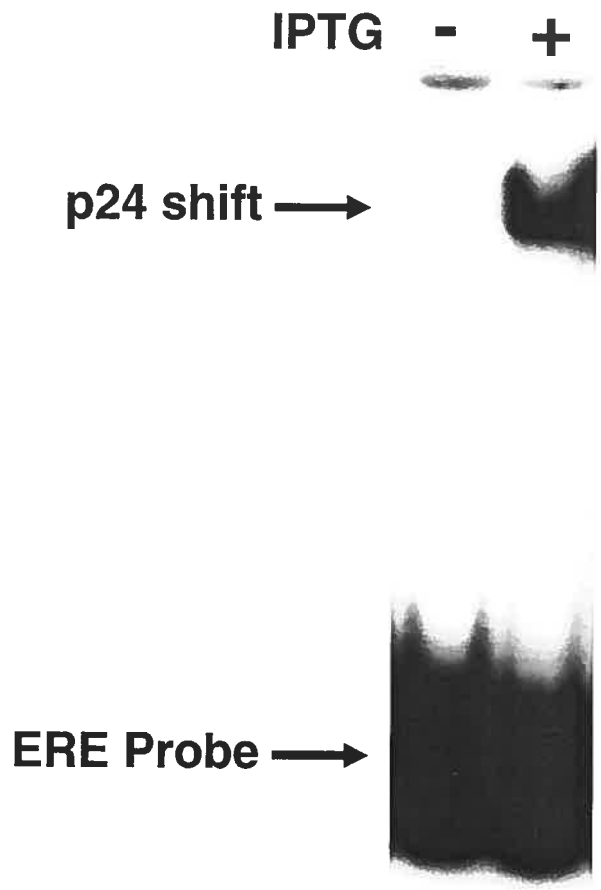
Interestingly, glutamine-rich domains have also been shown to bind ssDNA and to stabilize melted dsDNA [25]. Similarly, the p24 polyglutamine domain may help stabilize ssDNA in the ERE.

p24 is a DNA-binding protein

To confirm that p24 is a DNA binding protein, a truncated version of the p24 protein lacking the first 67 amino acids was expressed as a histidine tag fusion protein. An electrophoretic mobility shift assay (EMSA) with the purified recombinant protein showed that p24 can indeed bind the non-coding strand of the ERE (Figure 2.7). It was shown, using mutant versions of the NCS that recombinant p24 can bind ssDNA with the same sequence specificity as PBF-2 [16]. The recombinant protein was also used to produce antibodies and these were found to cross-react with a single 24 kD protein in purified PBF-2, and to inhibit the PBF-2 shift in an EMSA. These combined results show that p24 is the DNA binding component of PBF-2.

Figure 2.7

Recombinant p24 binds the ERE in vitro. EMSA showing the binding of recombinant p24 to a single-stranded ERE probe. The probe shift is observed only when the bacterial culture expressing p24 is induced with IPTG.


IPTG - +
p24 shift
ERE Probe

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Chapter III

Dual localization of p24 and plastid-nucleus communication

Introduction

The previous chapter has presented the cloning and initial characterization of *p24*, a gene coding for a 24 kD transcription factor found in potato tuber nuclei and involved in the activation of the nuclear gene *PR-10a*. Interestingly, the *p24* cDNA encodes a 30 kD polypeptide, suggesting that the protein is processed. Closer examination of the pre-sequence reveals that it contains a putative chloroplast transit peptide. Accordingly, three different protein localization prediction programs (PSORT [1], TargetP [2, 3] and Predotar [4]) concluded that the p24 protein should be localized in chloroplasts (Figure 3.1). When the predicted transit peptide sequence is removed and the remaining sequence is submitted to the PSORT program, the predicted localization is cytoplasmic (not shown). This appears to be in contradiction with previous results that demonstrate a role for p24 in the nucleus. While it does not possess a clear nuclear localization signal (NLS), p24 was initially purified from nuclei and it was shown that its binding to the ERE in the *PR-10a* promoter correlates with the activation of *PR-10a* gene expression [5, 6]. Furthermore, chromatin immunoprecipitation using anti-p24 antibodies showed that p24 is bound to the *PR-10a* promoter in vivo, in wounded and elicited tissues [6]. Together, these data would therefore suggest dual chloroplast/nucleus localization for p24.

Plastids refer to a group of organelles of which the photosynthesizing chloroplasts, the starch-storing amyloplasts, and the colourful chromoplasts of fruits are the best known. The plant plastid originated when a eukaryotic, mitochondria-possessing cell engulfed a photosynthetic cyanobacterium, more than a billion years ago. Since then, the endosymbiont lost its autonomy and most of its genome to the nucleus [7]. Today's plastids contain circular genomes of 120-160 Kb, containing approximately 130 genes [8]. A single leaf cell may contain up to 100 plastids, each harbouring approximately 100 identical copies of the plastid genome, for a total of up to 10 000 copies of the genome per cell [9]. This complexity, and the fact that many chloroplast proteins are now encoded by the nucleus demands a tight coordination of the

Figure 3.1

The full-length p24 protein is predicted to be targeted to chloroplasts. The p24 sequence is represented with the predicted transit peptide cleavage site at position 54 indicated with a blue triangle. Results from 3 intracellular localization prediction programs are indicated.

PSORT Results:

Chloroplast stroma	--- Certainty= 0.859
Chloroplast thylakoid membrane	--- Certainty= 0.491
Chloroplast thylakoid space	--- Certainty= 0.434
Microbody (peroxisome)	--- Certainty= 0.357

TargetP Results:

Chloroplast transit peptide	--- Probability= 0.964
Mitochondria transit peptide	--- Probability= 0.039
Signal peptide	--- Probability= 0.018
Other	--- Probability= 0.081

Probable length of transit peptide: 54 amino acids

Predotar Results:

Chloroplast	--- Probability= 0.733
Mitochondria	--- Probability= 0.002

activities in both compartments. The best example is perhaps that of ribulose bisphosphate carboxylase, the enzyme responsible for carbon fixation. The most abundant protein on earth is composed of two subunits. The large one (RbcL) is encoded by the chloroplast genome while RbcS, the small subunit, is encoded in the nucleus. Therefore, a mechanism must exist to ensure that both subunits are expressed at the same level. It was found that the presence of RbcS in the chloroplast influenced the translation of RbcL, through an unknown mechanism [10].

There is also a communication channel from chloroplasts to the nucleus. At least two known mechanisms are responsible for this communication. The first involves a precursor of chlorophyll, Mg-protoporphyrin IX which, when it accumulates, represses the expression of nuclear genes encoding chloroplast proteins [11]. The redox state of the chloroplast also has a strong effect on nuclear photosynthetic gene expression [12]. In both these cases, while the nature of the signal is known, the nature of the nuclear receptor is still elusive.

Dual localization of a protein in the nucleus and chloroplasts offers an attractive alternative for communication between the two compartments. The presence or absence of such a protein in a given compartment could act as a signal, or a post-translational modification of that protein in one compartment could theoretically communicate information to the other compartment. The latter assumes movement of the protein between the two compartments, rather than differential importation in each organelle. Alternatively, if a protein acts as a receptor for a given stimulus, the presence of that protein in two cellular compartments would allow for a coordinated response.

Dual localization has been reported before, though it is not a widespread phenomenon. The tobacco SigA2 protein, a putative chloroplast sigma factor involved in transcription, was found to have dual localization when fused to GFP [13]. However, the authors suggested that the nuclear localization of SigA2 was an artefact, as GFP itself can localize to the nucleus. If this were true, it would be expected that SigA2-GFP fluorescence would also be seen in the cytoplasm because GFP itself diffuses freely between the nucleus and the cytoplasm, but this was not the case. In addition, although only the first 184 amino acids of SigA2 were fused to GFP, this region contains two nuclear localization signals (NLS) and the program

PSORT predicts a nuclear localization for SigA2 with a certainty score of 0.940. The chloroplast stroma is the second predicted localization, with a score of 0.545. The authors were either unaware of these results, or they did not present them. If this is a true case of double-localization, it would be an interesting parallel to the case of p24, as sigma factors bind to specific DNA sequences in gene promoters and tether the plastid polymerase to these promoters.

Another example is pBrp, a transcription factor IIB (TFIIB)-related protein from *Arabidopsis*. pBrp is part of a plant-specific family of general transcription factors (GTF). In this case, pBrp was found on the cytoplasmic face of the chloroplast envelope and accumulated in the nucleus when the proteasome was inhibited [14]. The authors suggested that pBrp could be part of a retrograde signalling pathway from the chloroplast to the nucleus.

Finally, SEBF, a transcriptional repressor that binds the silencing element in the *PR-10a* promoter, also appears to have dual localization. Like p24, SEBF is synthesized as a precursor. It has a putative transit peptide and is predicted to be targeted to chloroplasts [15]. SEBF appears to be a single-copy gene in potato and alternative splicing was ruled out as a possible mechanism for producing two forms of the protein that would localize to the two different compartments. In cell fractionation experiments, SEBF was clearly localized in chloroplasts and nuclei [15], though the possibility that the antibodies also recognized a homologue of SEBF still cannot be completely excluded at this stage. The confirmation for the double localization of SEBF, however, came from laser-scanning confocal microscopy observations. An SEBF-GFP fusion could be seen in both compartments in transient and stable expression (Gidda S. and Joyeux A., unpublished). Furthermore, the protein detected in both compartments was of the same size, suggesting that the nuclear form of SEBF has lost the transit peptide [15]. While the role of SEBF in the chloroplast is not known, tobacco homologues (not known to be dually localized) are involved in RNA metabolism in the chloroplast [16, 17]. SEBF is a single-stranded DNA binding protein and it is possible that it too could have a role in RNA metabolism.

In animals, several proteins known to have a role in the nucleus were recently found to be present in mitochondria under certain conditions. For example, p53, a well-known transcription factor, is known to induce apoptosis

by activating the transcription of nuclear genes such as *Bax* and *Apaf-1*, and by repressing the transcription of *Bcl-2* [Reviewed in 18]. However, p53 also induces apoptosis via a transcription-independent pathway [19, 20]. It was shown that p53 can accumulate at the mitochondrial membrane [21] where it interacts directly with the protective BclXL and Bcl2 proteins, resulting in cytochrome c release [22]. Furthermore, artificially targeting p53 to mitochondria is sufficient to induce cell death [21]. Interestingly, p53 interacts with BclXL via its DNA-binding domain. In human tumour cells, naturally-occurring mutations in this domain inhibit the interaction with BclXL and the ability of p53 to cause apoptosis [22]. However, these same mutations cause constitutive accumulation of p53 at the mitochondria, while p53 is normally in the nucleus in non-induced conditions [22]. Hence it appears that, in addition to its role in the nucleus, the role of p53 at the mitochondrial membrane is crucial to promote apoptosis.

Similarly, the transcription factor TR3/Nur77/NGFIB induces apoptosis by relocating from the nucleus to the surface of mitochondria, where it induces cytochrome c release [23]. As for p53, the DNA-binding domain and transactivation function of TR3 are dispensable for its apoptotic function.

Apoptosis inducing factor (AIF) is another dually localized protein involved in apoptosis. Unlike p53, however, AIF has a mitochondrial localization signal (MLS), in addition to two NLS, and it translocates to the nucleus only upon apoptosis induction [24]. Once in the nucleus, AIF contributes to cell death by inducing large-scale DNA fragmentation. Interestingly, it possesses NADH oxidase activity and it is thought that this allows AIF to have a protective role in mitochondria under normal conditions [25, 26]. Accordingly, mouse harlequin (*Hq*) mutants have 80% less AIF and exhibit progressive degeneration of terminally differentiated cerebellar and retinal neurons, due to excessive accumulation of lipid hydroperoxides [26]. Furthermore WT AIF can rescue the increased oxidative stress-induced cell death in *Hq* cells [26]. Finally, the oxidase domain of AIF is dispensable for its apoptosis-inducing properties [25, 27]. Interestingly, it has recently been shown that mouse or human cells lacking AIF display a severe reduction in respiratory chain complex I activity, suggesting that AIF is involved in the biogenesis or maintenance of this protein complex [28].

It is not known whether the role of AIF in mitochondria is related to its role in the nucleus. It is probable however that the translocation of AIF away from mitochondria during apoptosis increases oxidative stress and therefore induces further signalling to the nucleus to promote cell death [29]. AIF can associate with DNA [30], and deletions that interfere with this property also abolish the apoptosis-inducing capabilities of AIF [31]. This suggests that AIF induces apoptosis by associating with DNA. How AIF promotes DNA degradation is not clear however, as it does not possess any intrinsic nuclease activity [32]. Interestingly, another protein that translocates from mitochondria to nuclei upon apoptosis is endonuclease G [33]. In *Caenorhabditis elegans* at least, the Worm AIF homologue (WAH-1) interacts directly and cooperates with the endonuclease G orthologue CPS-6 (ced-3 protease suppressor 6) in a complex that has been termed the "degradosome" [32]. It is therefore possible that the nuclear role of AIF during apoptosis is to tether endonuclease G to DNA.

Dual localization of p24 in chloroplasts and the nucleus is therefore not inconceivable. If p24 is localized in two cellular compartments however, what is the role of this dual localization? Given the role of p24 in the activation of defence genes in the nucleus, we could expect that the role of p24 in the chloroplast would be related to defence and that p24 could coordinate the activities of these two compartments during the plant defence response. Alternatively, the roles of p24 in these two compartments may be antagonistic, such as appears to be the case for AIF.

This chapter presents the dual localization of p24 in chloroplasts and the nucleus. In chloroplasts, p24 is involved in the wound-induced activation of a gene involved in photosynthesis. This, in turn, generates a signal that is perceived outside the chloroplast and results in altered nuclear gene expression. These results are discussed in the larger context of cellular defence.

Materials and methods

Plant material

Potato (*Solanum tuberosum* cv Kennebec) and tobacco (*Nicotiana tabacum*) plants were grown in an environmental growth chamber (Conviron) under long-day (16h) photoperiod conditions [see 15]. In vitro plants were

also grown under 16h photoperiod in standard MS medium (Sigma) and transplanted to fresh medium every four weeks [see 15].

Plasmid constructs and plant transformation

The p24-GFP construct was produced as follows: the coding sequence from the Emerald GFP (Clontech) was excised from the pGFP plasmid using the *Bam*H1 and *Xba*1 enzymes and inserted into pBluescript (Stratagene). The *p24* sequence, including the 5' untranslated region (134 nucleotides, see Accession AF233342), was inserted in frame 5' of the GFP. The p24-GFP fusion was then inserted into the pBin19 binary vector containing a double 35S cauliflower mosaic virus (CaMV) and a NOS terminator [34]. The CT-GFP vector [35] was a gift from M.R. Hanson, Cornell University.

The p24 sense construct was produced by excising the p24 cDNA sequence from the pBluescript plasmid using the enzymes *Bam*H1 and *Kpn*1 and inserting it into a pBIN19 binary vector containing a double 35S cauliflower mosaic virus (CaMV) and a NOS terminator. For the antisense construct, the p24 coding sequence was amplified with the primer 5'-TGCTTGTAGCGGTACCAGAAC and the pBluescript Reverse primer. The DNA fragment was then inserted into the *Kpn*1 and *Sac*1 of the pBIN19 vector. All constructs were electroporated into *Agrobacterium tumefaciens* LBA4404. Leaf discs were transformed as described [36]. Transformed plants were selected by including 50 mg/L Kanamycin in regeneration medium. Three transgenic potato lines (S1, S2 and AS) and two transgenic tobacco lines (p24-GFP and CT-GFP; 1 line of each) were selected and used throughout this thesis.

For transient expression, the p24-GFP fusion was extracted from the pBin19 vector and cloned into the pBI223 vector [6]. Leaf mesophyll protoplasts were isolated and transformed as described [5].

Protein purification

Purified potato chloroplast and nuclear fractions for protein gel blot analysis were isolated as described [15]. For tobacco, 10 g of leaf tissue or 8 g of root tissue were frozen in liquid nitrogen and ground in 1 mL of NEBH buffer (12.8% (v/v) hexylene glycol, 10 mM Pipes-KOH pH 6.0, 0.15 mM spermine, 10 mM $MgCl_2$, 0.5 mM spermidine, 20 mM beta-mercaptoethanol).

After centrifugation for 5 min at 4 000 g at 4°C, the pellets were resuspended in 10 mL (leaf samples) or 8 mL (root samples) of NP40 buffer (10 mM MES-NaOH pH 6.0, 260 mM sucrose, 10 mM NaCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 14.3 mM beta-mercaptoethanol, 0.1 % (w/v) bovine serum albumin, 1% (v/v) Nonidet-P40). The extracts were centrifuged for 10 min as above and the supernatants were frozen at -80°C in 10 % (v/v) glycerol (chloroplast fraction). The pellets were then washed four times in 25 mL NP40 buffer. The final pellets were resuspended in 3 mL (leaf samples) or 2.44 mL (root samples) lysis buffer (20 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA). The nuclei were lysed by sonication, incubated for 30 min on ice, and centrifuged for 45 min at 12 000 g at 4°C. The supernatants were frozen at -80°C in 10 % (v/v) glycerol (nuclear fraction).

Laser-scanning confocal microscopy

GFP fluorescence (500-530 nm) was visualised with a Leica DM IRB/E laser-scanning confocal microscope using a 488 nm laser excitation source. Chlorophyll autofluorescence was visualised at 650-700 nm. Hand-made thin sections of fresh leaves or roots from in vitro grown plants were placed in sterile water on a slide, under a cover slip sealed with nail varnish, and examined directly. Protoplasts were visualised by placing a drop of protoplast solution on the slide, covering with a cover slip, and sealing with nail varnish. For DNA staining, protoplasts were incubated with 5 µM Syto85 (Molecular Probes) for 10-30 min at room temperature, washed with fresh culture medium, and examined right away. Syto85 fluorescence (570-600 nm) was visualised using a 568 nm laser excitation source. GFP and Syto85 fluorescence images were collected sequentially, and no fluorescence cross-talk was observed under our conditions (not shown). Pseudocoloring of the images, maximal projections, and image overlays were done using the Leica confocal software (LCS).

Search for PB sites

A program was designed to screen the published sequence of the tobacco chloroplast genome (accession NC_001879) for potential p24 binding sites. This Perl computer program can be found in Annex 1.

DNA immunoprecipitation

Tobacco leaves (10 g) from wild-type (WT) and a p24-GFP transgenic plants were harvested, de-veined, and placed overnight in the dark at 4°C. Tissues were fixed for 15 min in 1% (v/v) formaldehyde, rinsed in distilled water and blotted dry. Chloroplasts were isolated and purified on a 40/80% (v/v) two-step percoll gradient as described [37] and lysed osmotically in immunoprecipitation buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate) supplemented with a cocktail of protease inhibitors (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged for 5 min at 12 000 g at 4°C, and filtered through glass wool. Chloroplast DNA was sonicated twice for 20 s, resulting in ~1kb DNA segments, and immunoprecipitated as described [6] except that protein G-agarose (Santa Cruz Biotechnology) was used. The antibodies used (4µg of each) were anti-GFP (Roche), and mouse pre-immune serum (Sigma). A 300 bp fragment of the *Ycf3* promoter (accession Z00044), containing a PB element, was amplified using the primers 5'-GTAGCAATCCATTCTAGAAT and 5'-TCTTTGTAATTT-GTATCATGAT. A 300 bp control region of chloroplast DNA not containing a PB (position 5853, accession NC_001879) site was amplified using the primers 5'-ATCGAAAAAGTTTGATCAATTC and 5'-GTTGTGGATTTGTACATCCA. The polymerase chain reaction (PCR) conditions were 5 min initial denaturation at 94°C followed by 25 cycles of 45 s at 94°C, 1 min at 50°C, and 1 min at 72°C. This was followed by 5 min at 72°C. For the control region, the cycling conditions were extended for another 5 cycles, in order to ensure that no DNA fragment had been immunoprecipitated with the anti-GFP antibodies.

Gene expression analysis

Leaf RNA samples from WT and p24 transgenic potato plants were isolated using TRIZOL (Invitrogen). For RNA gel blot analysis, the *Ycf3* (accession Z00044) and *Fed1* (accession AJ307031) genes were amplified from tobacco and potato DNA respectively, and random-primer labelled with α^{32}P-dCTP. The *Ycf3* gene was amplified using the primers 5'-CCTAGATCACGGATAAATGGAA and 5'-CTTCAACCAATTA-TGCGCTTCA. The *Fed1* gene was amplified using the primers 5'-CTGGTACCATGATT-

AGCACT and 5'-GAAAAGTAAATGCTCATGAAAC. RNA hybridization and detection was performed according to standard methods [38].

To analyze the expression of *p24* in WT and transgenic potato plants, a protein gel blot was performed on total protein extracts with the anti-p24 antibodies (dilution 0.001). The antibody-antigen interaction was revealed using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to manufacturer instructions [also see 15].

Chlorophyll fluorescence analysis

Leaf segments (3 cm x 3 cm) were cut from the terminal leaflet of the 7^{th} leaf of WT and *p24* transgenic potato plants. The segments were floated on water or water + 10 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in Petri dishes and incubated in a growth chamber (Conviron) for 24 hours, under 16-hour photoperiod. Alternatively, photosynthesis was measured from fresh leaves directly on the plant. Following 30 min of dark-adaptation, chlorophyll *a* fluorescence induction was analyzed using a pulse amplitude-modulated (PAM) fluorometer (FMS, Hansatech Instruments) as described [39]. The saturating flash and modulated lights were of 700 and 1 µmol photons m^{-2} s^{-1} respectively. The actinic light intensity was 330 µmol photons m^{-2} s^{-1} for the fresh and wounded treatments and 65 µmol photons m^{-2} s^{-1} for the wounded + DCMU treatment. The experiment was repeated twice with different plants. On each occasion, three readings were done for each treatment and each transgenic line. ΦPSII was calculated as $(Fm'-F_s)/F_m'$, where F_m' is maximum fluorescence in the light and F_s is steady-state fluorescence. Electron Transport Rate (ETR) was calculated as ΦPSII x actinic light intensity.

Results and discussion

p24 dual localization

The apparent contradiction between the predicted chloroplast localization of p24 and its demonstrated role in the nucleus prompted us to examine the functional relevance of the transit peptide. A protein gel blot of chloroplast and nuclear fractions from potato leaves showed that p24 is present in both compartments (Figure 3.2a). To ensure that this dual localization did not result from two different genes, we produced tobacco plants expressing a p24-GFP fusion under the control of a constitutive promoter. As a control, we used plants overexpressing a transit peptide-GFP fusion (CT-GFP) (with the same promoter) targeted only to plastids [35]. A protein gel blot with anti-GFP antibodies showed p24-GFP in both compartments while CT-GFP, as expected, was found mainly in plastids (Figure 3.2b). No band was detected with the anti-GFP antibodies in untransformed plants (data not shown). The presence of a small amount of CT-GFP in the nuclear fraction is probably due to contamination by the plastid fraction as RbcL, the large subunit of the Rubisco enzyme normally present exclusively in chloroplasts, was also detected in the nuclear fraction of CT-GFP plants. All bands detected were of expected sizes (RbcL ~52.5 kD, Cdc2 ~32 kD, H1 ~29.8 kD).

It should be noted that the relative abundance of p24 in both compartments cannot be estimated from these blots. The same amount of protein was loaded in each lane but the total protein concentration differs in chloroplasts and nuclei. In fact, it was estimated that nuclear proteins are over-represented by a factor of 55 in these blots (data not shown). If the protein quantities in each lane are adjusted for this dilution factor, p24 is detectable in chloroplasts but only barely in nuclei (data not shown).

The dual localization of p24 was confirmed by laser-scanning confocal microscopy (LSCM) (Figures 3.3 and 3.4). Figure 3.4a shows the presence of p24-GFP in both the nucleus and the surrounding plastids in roots, while the fluorescence in CT-GFP plants was only detected in plastids (Figure 3.4b). However, in most cells, p24-GFP fluorescence was difficult to distinguish above background in the nucleus and was only seen in plastids. The latter

Figure 3.2

Dual localization of p24. **A** Detection of endogenous p24. Protein gel blot with purified plastid (Pl) and nuclear (N) fractions from potato leaves. The antibodies used are as indicated. Each lane contains 40 μg of protein. **B** Detection of GFP-tagged p24 in tobacco leaves and roots. Protein gel blot of purified plastid (Pl) and nuclear (N) fractions from p24-GFP and CT-GFP tobacco plants. The antibodies are as indicated. Each lane contains 5.6 μg of root protein or 17.5 μg of leaf protein.

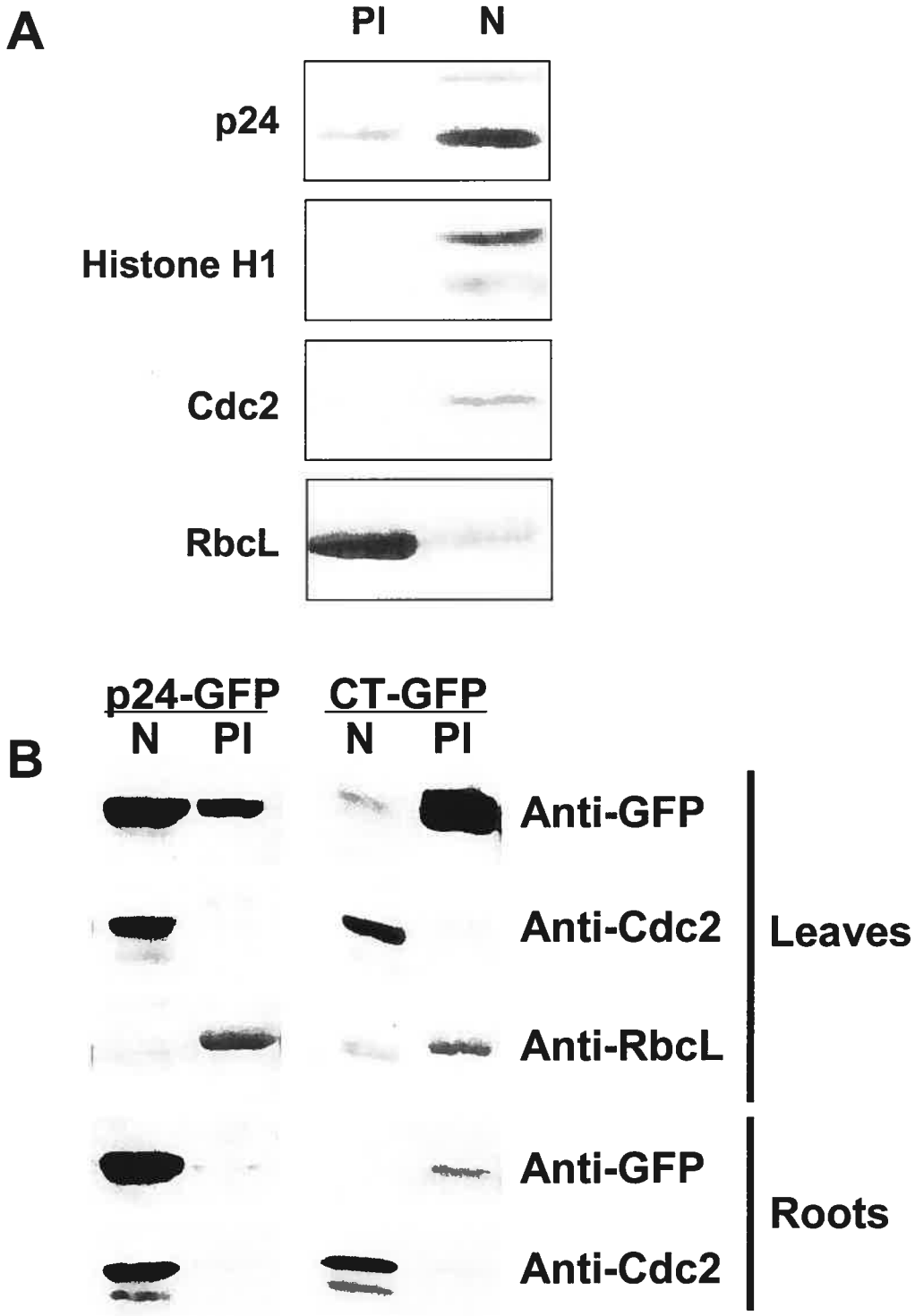
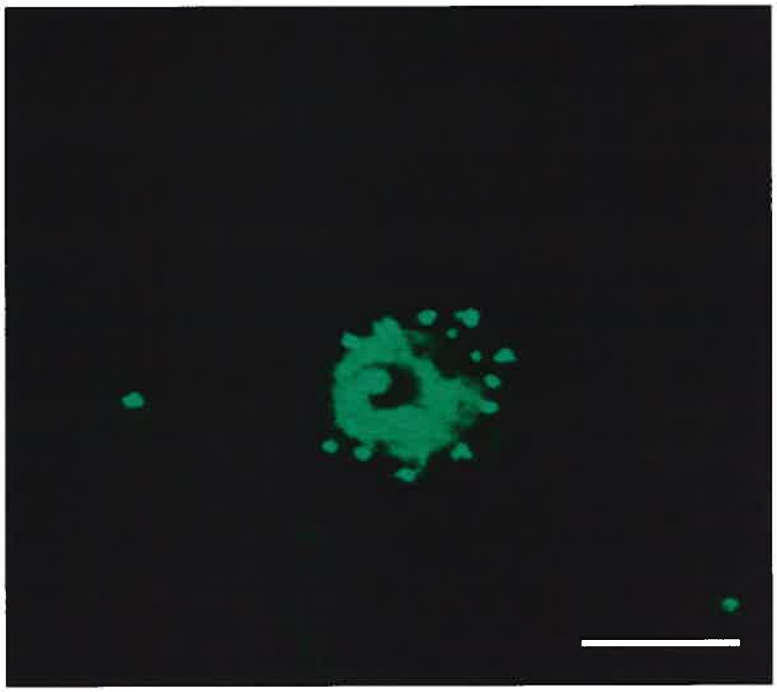

A
Pl
N
p24
Histone H1
Cdc2
RbcL
B
p24-GFP
CT-GFP
N
Pl
N
Pl
Anti-GFP
Anti-Cdc2
Anti-RbcL
Leaves
Anti-GFP
Anti-Cdc2
Roots

Figure 3.3

LSCM of a p24-GFP tobacco root. Upper panel is GFP fluorescence pseudo-coloured in green. Bottom panel is corresponding phase-contrast image. Scale bars = 8 μm.



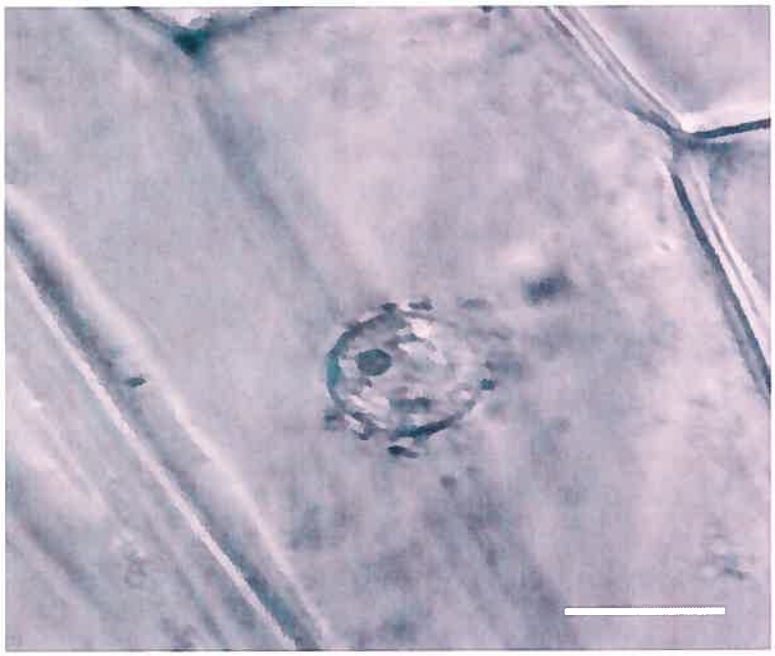
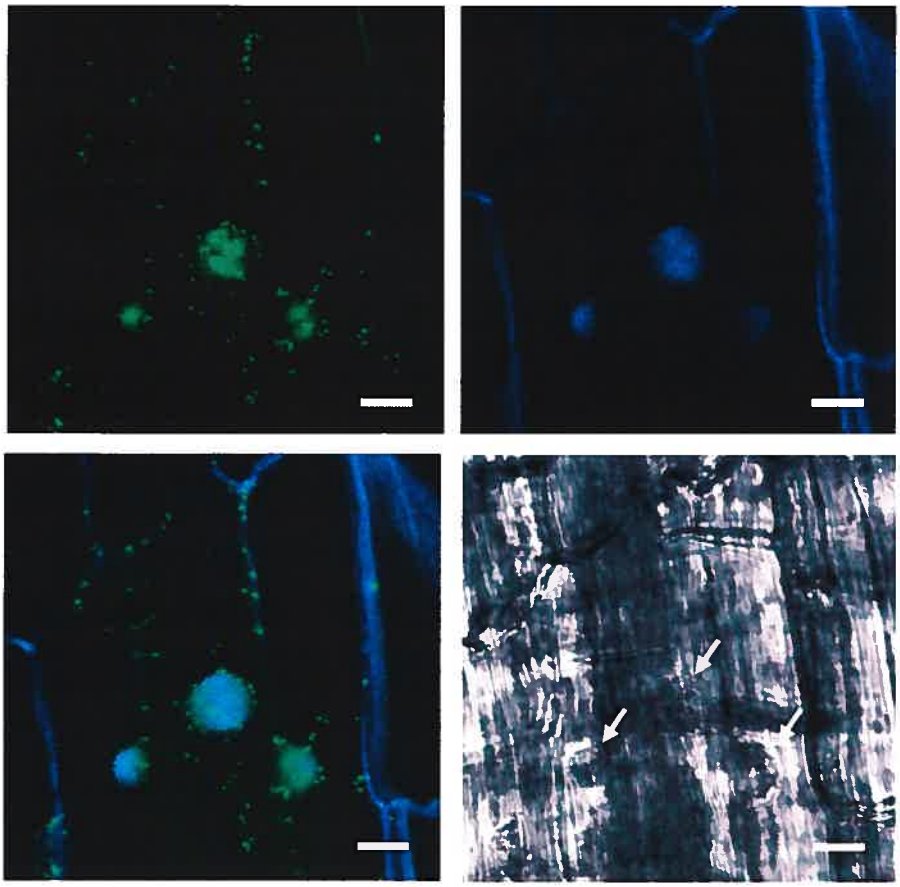


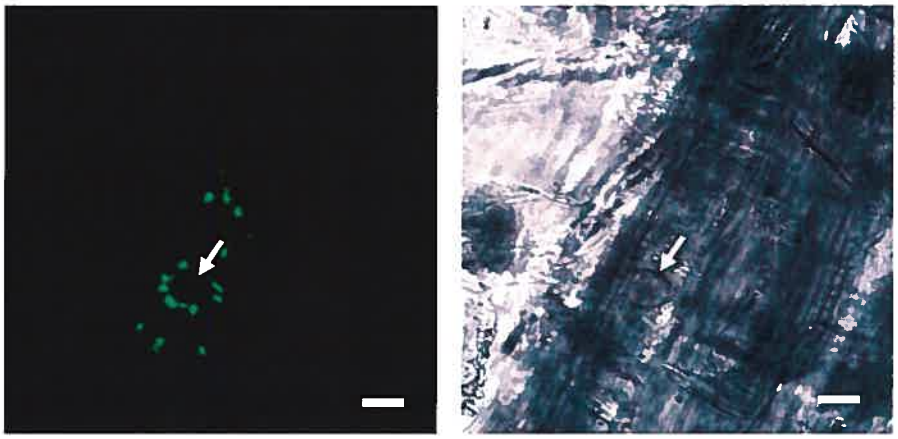
Figure 3.4

A Laser scanning confocal microscopy (LSCM) of p24-GFP tobacco roots stained with the fluorescent DNA dye Syto85. The upper left panel is GFP fluorescence pseudo-coloured in green, the upper right panel is Syto85 fluorescence pseudo-coloured in blue, the bottom left panel is the overlay of GFP and Syto85 fluorescence, and the bottom right panel is the corresponding phase-contrast image. **B** LSCM of CT-GFP tobacco roots. The upper panel is GFP fluorescence and the bottom panel is the corresponding phase-contrast image. Arrows indicate the nuclei. Maximum projections are shown. Scale bars = 8 μm.

A



B



observation is difficult to reconcile with the biochemical data. As mentioned however, the quantity of p24 protein in nuclei is probably overestimated in the protein gel blots, and it is possible that there is not enough p24-GFP in most nuclei to be detectable by confocal fluorescence microscopy. This would imply that, either nuclear p24 is being degraded post-translationally, or that only a small number of the p24 proteins are being imported in most nuclei. In accordance with the confocal results, there was relatively more p24-GFP in the nuclear fractions of tobacco roots vs. leaves, compared to the plastid fractions (Figure 3.2b).

The reason for a larger abundance of p24 in some root nuclei is unknown. The human AIF protein is translocated from mitochondria to the nucleus only upon apoptosis [24]. Likewise, p24 might be imported into nuclei under certain specific conditions. Previous biochemical experiments however have suggested that PBF-2, the protein complex containing p24, is always present in nuclei, albeit in small quantities [5]. While PBF-2 activity can only be detected in potato tuber crude nuclear extracts after elicition, the same activity can be detected in fresh potato tuber nuclei after purification. This suggests that PBF-2 is not imported into nuclei exclusively upon elicitation. While these results may not be applicable to all cells, they are in agreement with the biochemical data presented in Figure 3.2. This does not exclude the possibility that, under certain conditions, p24 is needed in larger quantities in the nucleus, as suggested by the confocal data.

p24 is a chloroplast DNA-binding protein

Examination of p24-GFP fluorescence in transgenic leaf cells indicated that p24 is present in speckles within chloroplasts (Figures 3.5). Optical sections through leaf cells showed that at least some of the p24-GFP speckles are inside the chloroplasts, and not at the periphery (Figure 3.6). While p24-GFP is presented in guard cells only, fluorescent speckles could be observed in most leaf cells.

These speckles are reminiscent of nucleoids, DNA-protein complexes that contain the plastid genome. Furthermore, most p24-GFP speckles appear to be associated with the chloroplast stroma, rather than the

Figure 3.5

Localization of p24 with in chloroplast speckles. LSCM of p24-GFP tobacco leaf guard cells. Upper left panel is maximal projection of chlorophyll autofluorescence pseudo-coloured in red. Upper right panel is maximal projection of GFP fluorescence pseudo-coloured in green. Bottom left panel is overlay of chlorophyll and GFP fluorescence. Bottom right panel is corresponding phase-contrast image. Scale bars = 8 μm.

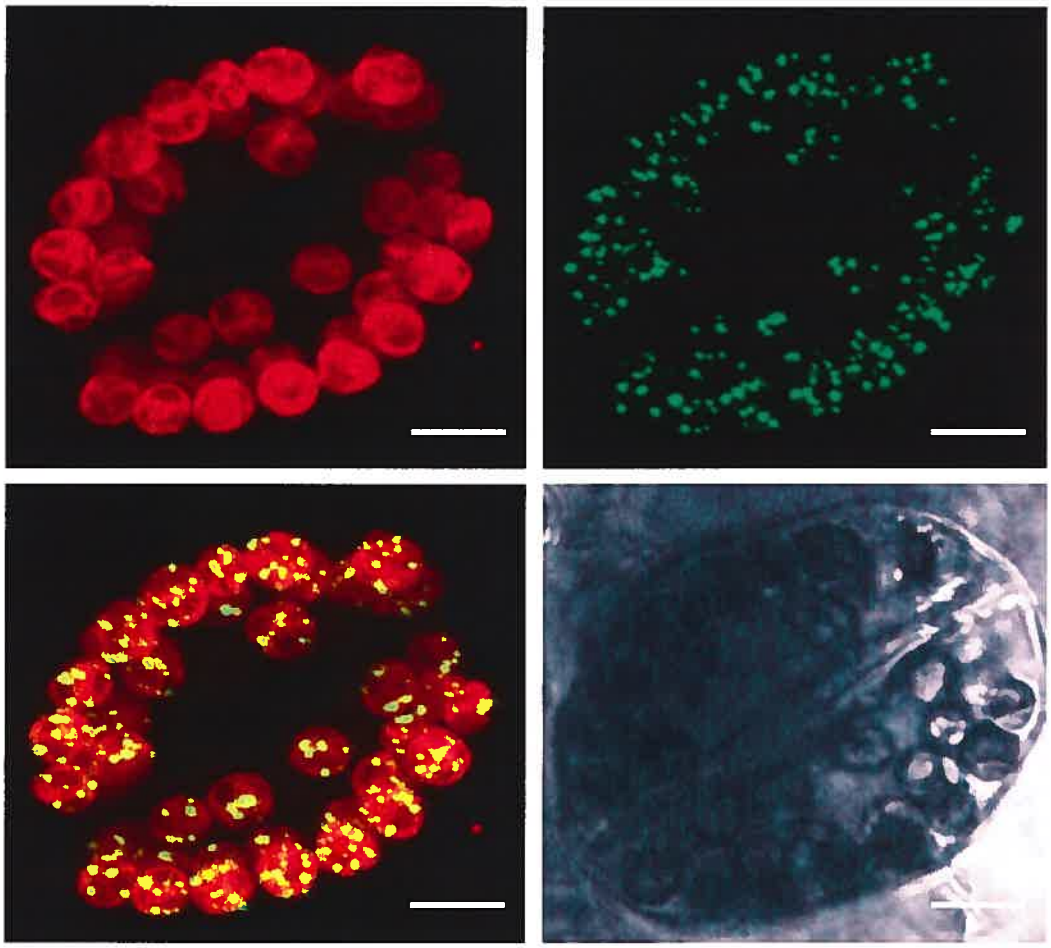
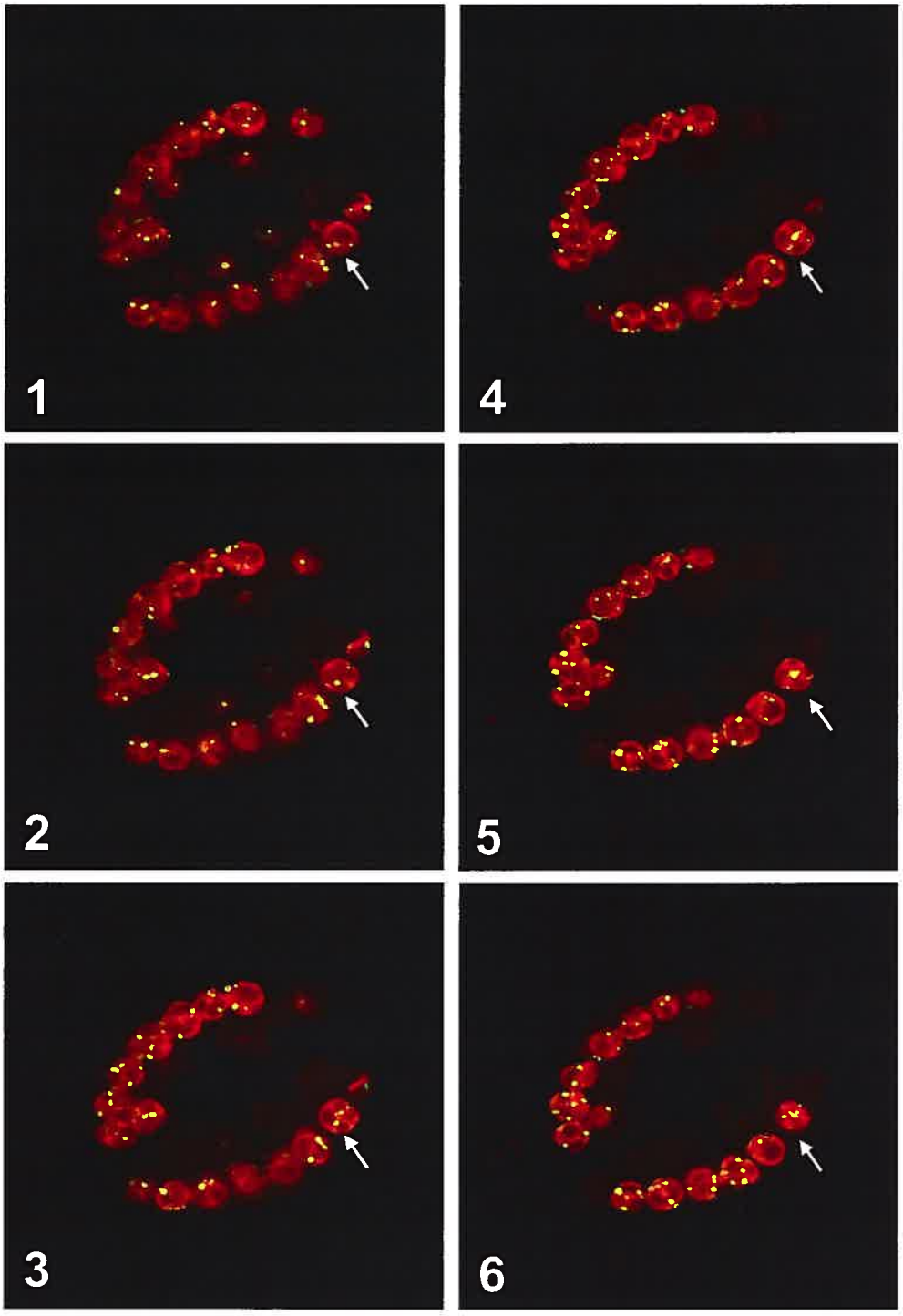


Figure 3.6

p24-GFP is localized inside chloroplasts. Laser scanning confocal microscopy (LSCM) was used to collect optical sections (0,5 μm between each section) through a p24-GFP transgenic tobacco leaf guard cell. Six consecutive sections are shown numbered 1-6. GFP fluorescence was pseudo-coloured in green, while chlorophyll autofluorescence was pseudo-coloured in red. The images were overlaid using the Leica confocal software. The arrow indicates a speckle of p24-GFP fluorescence that appears in the second section and disappears in the fourth section, indicating that it is inside the chloroplast.


1
4
2
5
3
6

fluorescent chlorophyll-containing thylakoid membranes (Figure 3.7). These results, combined with the fact that p24 is a DNA-binding protein [5], suggested that p24-GFP could be bound to chloroplast DNA.

Co-localization of Syto85, a fluorescent DNA dye, with p24-GFP in chloroplasts of tobacco mesophyll protoplasts (Figure 3.8) confirmed that p24 is associated with DNA. This was more easily observed in protoplasts, as the Syto85 dye did not appear to penetrate the cell wall of plant cells in leaf tissues. It should be noted once again that, while the nucleus was visible with Syto85, p24-GFP was only seen in the chloroplasts of the leaf protoplasts observed (Figure 3.9).

p24 binds the chloroplast *Ycf3* promoter in vivo

We next examined whether p24 binds chloroplast DNA in vivo. Eighteen putative p24-binding sites (PB element; GTCAAAAA) are present in the tobacco chloroplast genome (Table I). One of these sites lies in the promoter of the *Ycf3* gene and is conserved in many *Ycf3* proximal promoters, including that of potato *Ycf3* (Figure 3.10), suggesting that the PB element plays an important role in the expression of this gene.

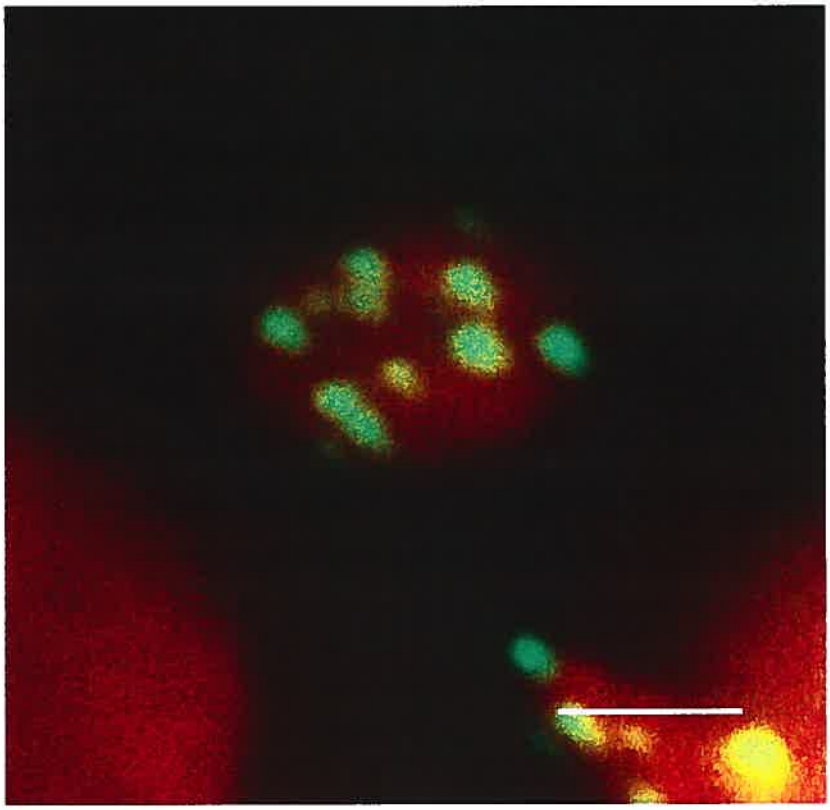
A chromatin immunoprecipitation method was adapted to chloroplasts (Figure 3.11) to test whether p24 is bound to chloroplast DNA in vivo. The *Ycf3* promoter, but not a DNA fragment exempt of p24-binding sites, was immunoprecipitated from p24-GFP tobacco chloroplast DNA with anti-GFP antibodies (Figure 3.12). In WT plants, the *Ycf3* promoter could not be immunoprecipitated with anti-GFP antibodies. Together, these results confirmed that p24 binds chloroplast DNA in vivo, and with sequence specificity.

Ycf3 transcription is p24-dependent in wounded tissues

p24 is a transcriptional activator in the nucleus [6] and binding of p24 to the promoter of *Ycf3* raises the interesting possibility that it plays a similar role in chloroplasts. However, RNA gel blot analysis of *Ycf3* in fresh leaves of potato plants over- and underexpressing p24 (Figure 3.13) showed only a small difference in *Ycf3* expression (Figure 3.14a). This indicates that p24 does not play a significant role in *Ycf3* expression in fresh tissues. The presence of several bands in Ycf3 RNA gel blots is explained by the fact that this gene is part of a gene cluster containing *Ycf3*, *psaA*, *psaB* and *rps14* that

Figure 3.7

p24 is associated with the chloroplast stroma. LSCM of an isolated chloroplast from a p24-GFP transgenic tobacco plant. The upper panel shows an overlay of the GFP image (green) with the chlorophyll fluorescence image (red). The bottom panel shows an overlay of the GFP image (green) with the corresponding phase contrast image. Scale bars = 1.44 μm.



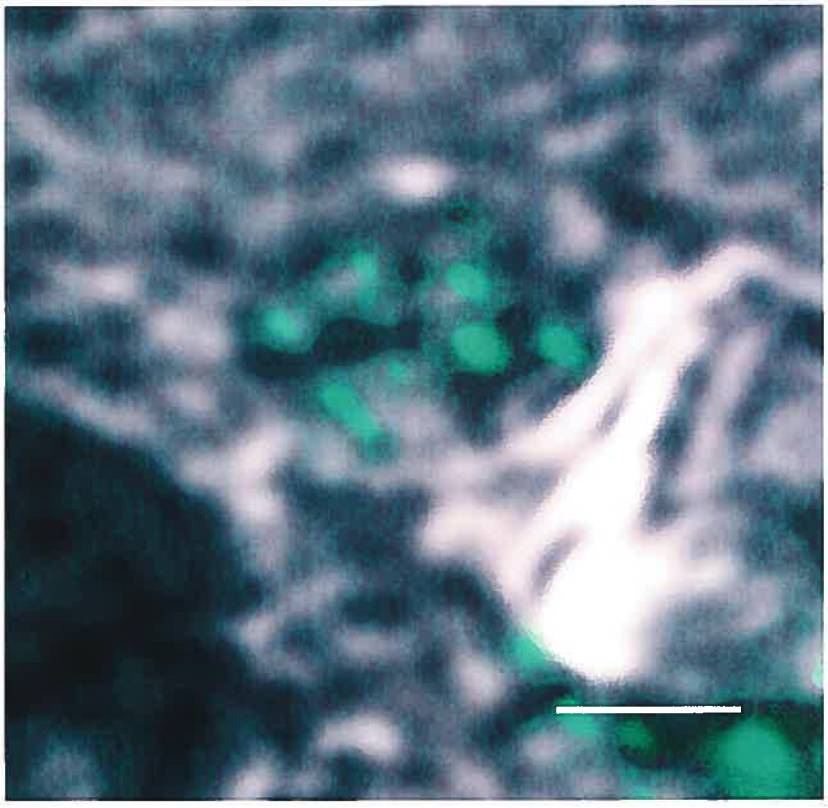


Figure 3.8

LSCM of a tobacco leaf mesophyll protoplast transiently expressing p24-GFP and stained with the DNA dye Syto85. First panel is maximal projection of chlorophyll autofluorescence pseudo-coloured in red. Second panel is maximal projection of GFP fluorescence pseudo-coloured in green. Third panel is maximal projection of Syto85 fluorescence pseudo-coloured in blue. Bottom panel is overlay of all three images. Scale bars = 8 μm.

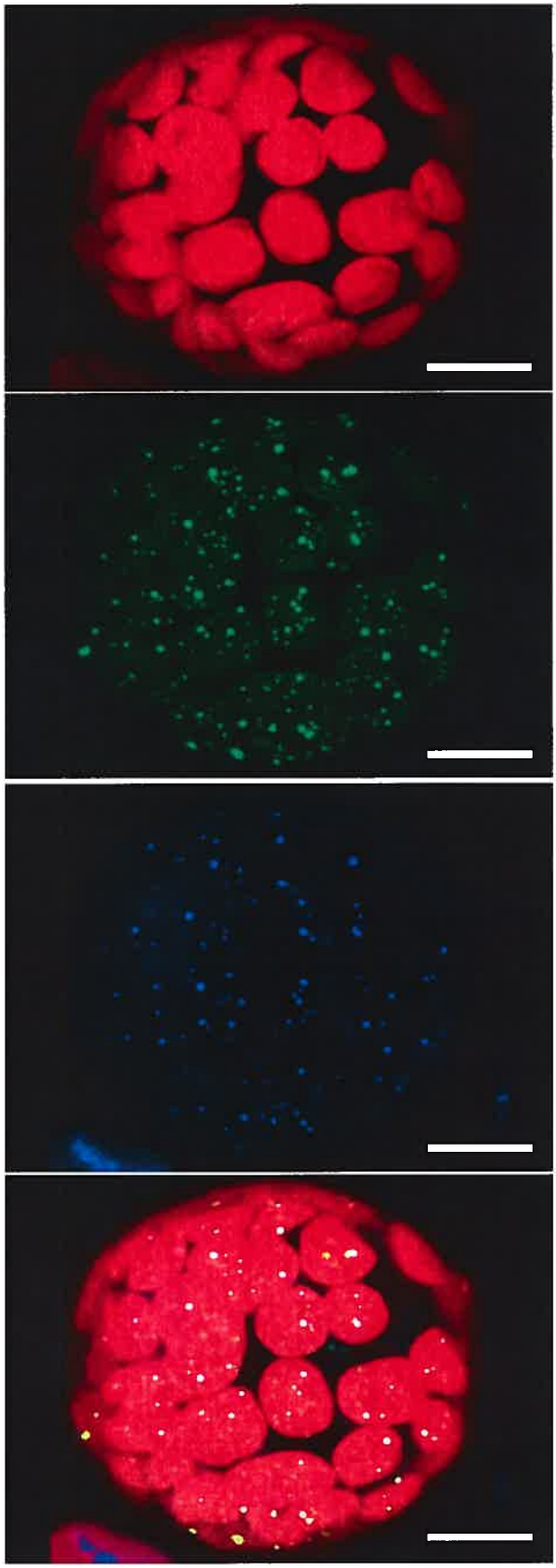

Chlorophyll autofluorescence
p24-GFP
Syto85
Overlay

Figure 3.9

Single optical section of a tobacco mesophyll protoplast transiently expressing p24-GFP. The protoplasts were incubated with the fluorescent DNA stain Syto85. The top left panel shows GFP fluorescence pseudo-coloured in green. The top right panel shows Syto85 fluorescence pseudo-coloured in blue. The bottom left panel shows chlorophyll autofluorescence pseudo-coloured in red. The bottom right panel shows the overlay of all three images. Scale bar = 8 μm.

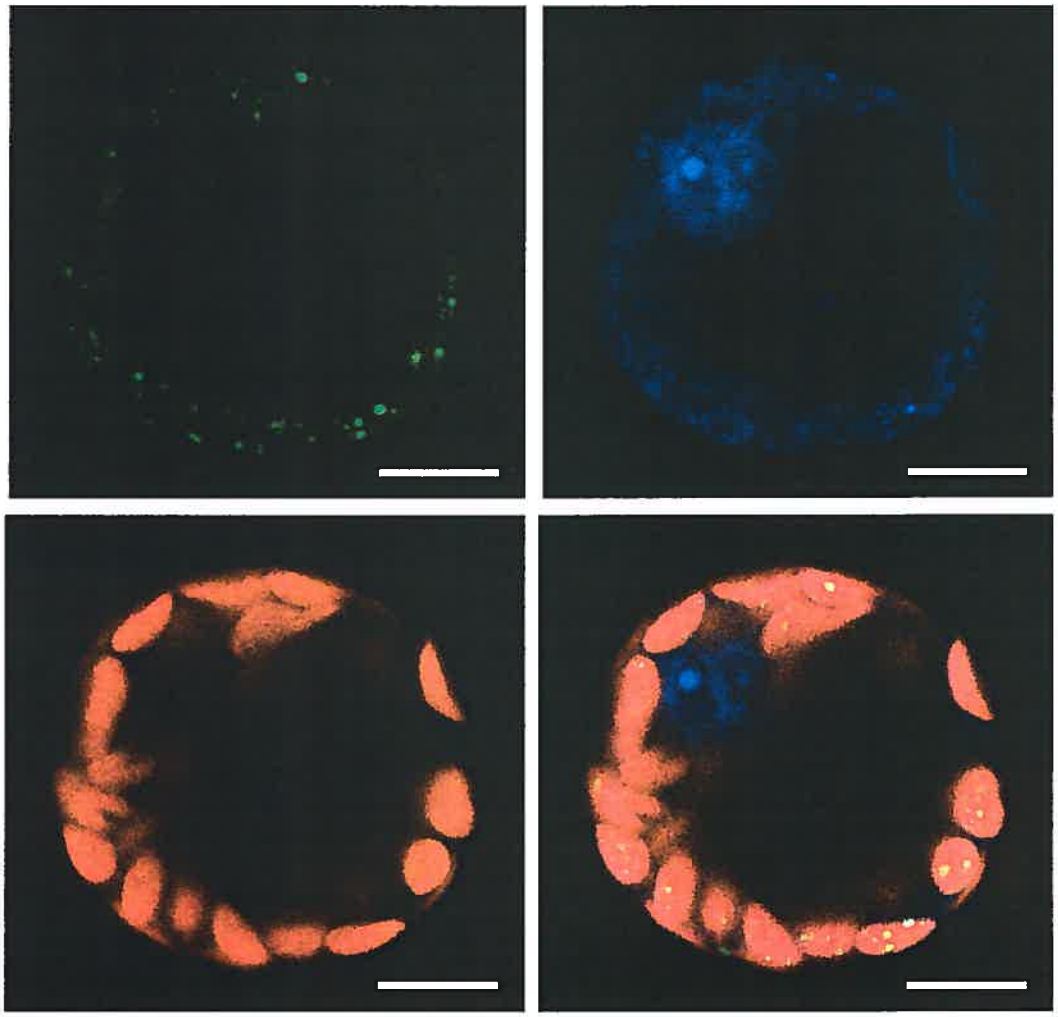


Table I

Table of potential PB sites in tobacco chloroplast genome. The description, position and DNA strand of each PB site were derived from the annotation of the tobacco chloroplast genome (accession NC_001879).

Table of potential PB sites in tobacco chloroplast genome

POSITION	STRAND	DESCRIPTION
221	+	Between *tRNA-His* & *psbA*
13550	+	Between *atpF* & *atpH*
92676	+	Inside *Ycf2*
114366	+	-695 of ATG of *rpl32*
123828	+	Inside first exon of *ndhA*
19622	-	Inside *rpoC2*
29443	-	Between *tRNA-Cys* & *Ycf6*
31297	-	Between *psbM* & *tRNE*
45018	-	Inside first intron of *Ycf3*
46417	-	In promoter of *Ycf3*
59190	-	219 bp after *RbcL*
70944	-	Betwen *rps18* and *rpl20*
82349	-	Inside initiation factor 1
113761	-	Inside *ndhF* (same), between *ars1* & *rpl32*
122184	-	Inside last exon of *ndhA*
125292	-	Inside *rps15*
126890	-	Far upstream of *rps15*
149940	-	Inside *Ycf2*

Figure 3.10

The p24-binding (PB) site in *Ycf3* promoters is conserved in plant chloroplast genomes. Arrows indicate the position of the PB element relative to the ATG. Accession numbers are as follow: Tobacco NC_001879, Potato DQ231562, Spinach NC_002202, Rice NC_001320, Maize NC_001666, Wheat NC_002762, Arabidopsis NC_000932, Mustard AJ242660.

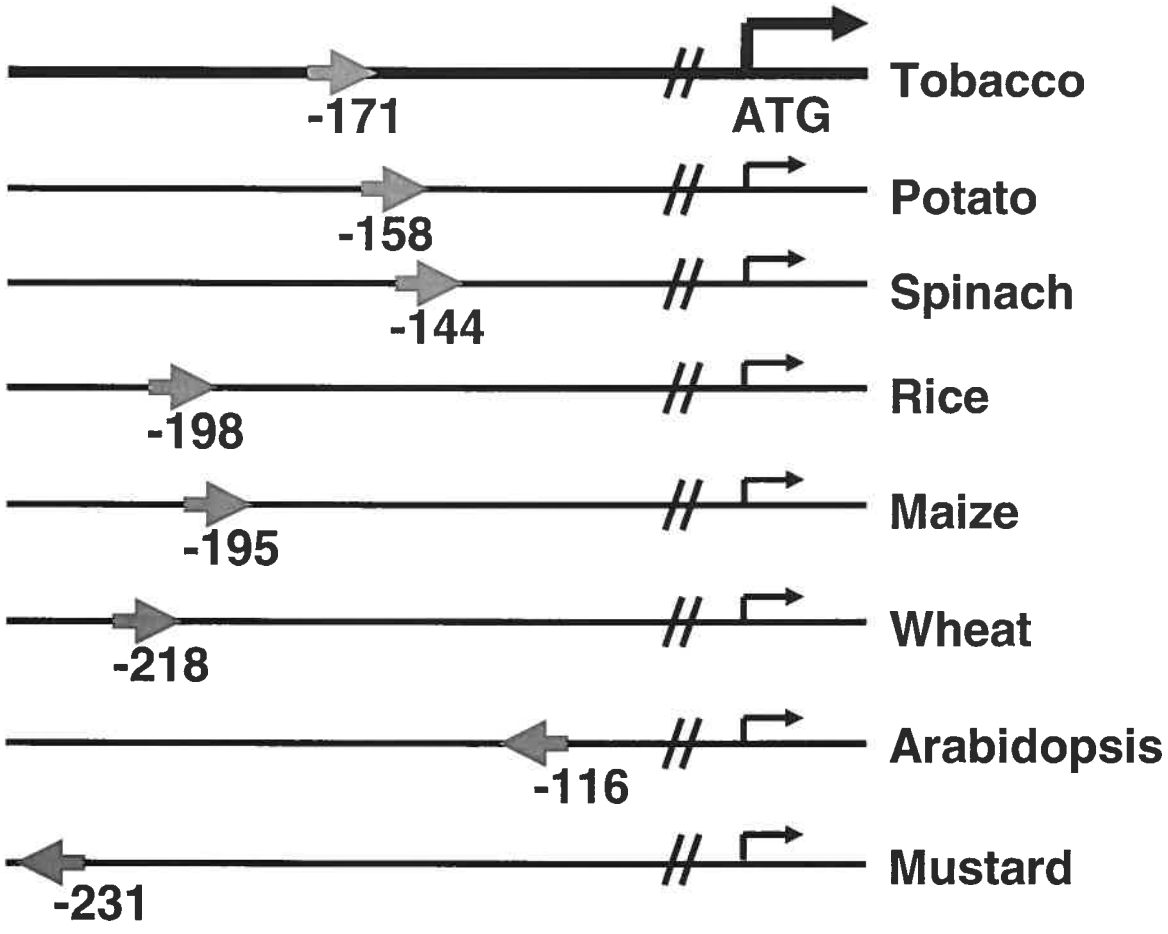

-171
ATG
Tobacco
-158
Potato
-144
Spinach
-198
Rice
-195
Maize
-218
Wheat
-116
Arabidopsis
-231
Mustard

Figure 3.11

The chloroplast DNA immunoprecipitation method. Leaf tissues from WT and p24-GFP transgenic tobacco plants are fixed in formaldehyde. Chloroplasts are isolated on a Percoll gradient and then lysed. The DNA is extracted and sheared. Protein-DNA complexes are immunoprecipitated with anti-GFP antibodies. The cross-links are reversed and the DNA-binding sites are amplified by PCR.

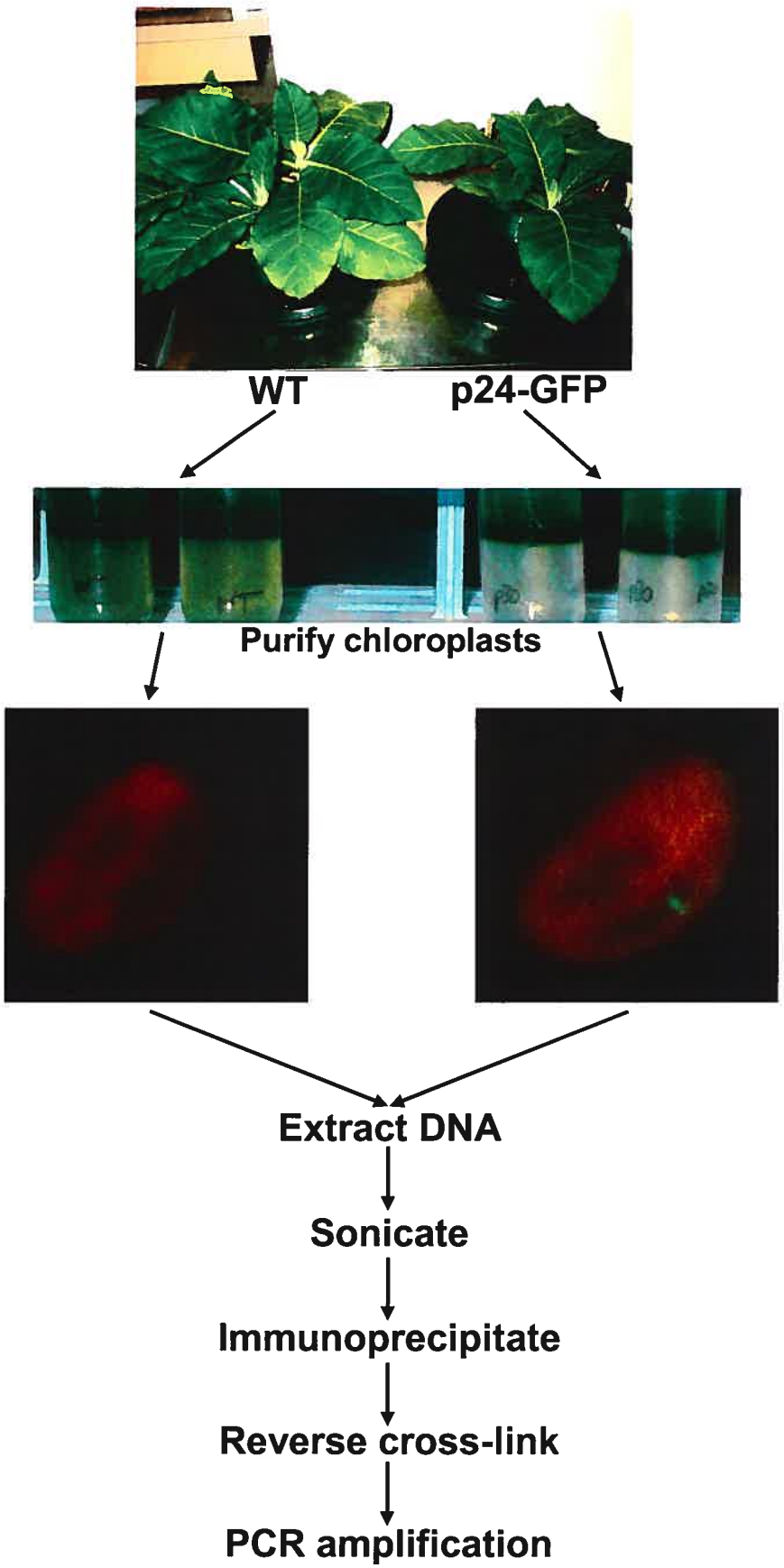

WT
p24-GFP
Purify chloroplasts
Extract DNA
Sonicate
Immunoprecipitate
Reverse cross-link
PCR amplification

Figure 3.12

p24 binds the *Ycf3* promoter in vivo. DNA immunoprecipitation of chloroplast *Ycf3* promoter with anti-GFP antibodies in wild-type (WT) and p24-GFP tobacco plants. Input is the extracted DNA prior to immunoprecipitation. DNA immunoprecipitation was also performed with the p24-GFP plants using a pre-immune serum (Pre-Immune). The same experiment was performed with a region of chloroplast DNA containing no PB site (No PB).

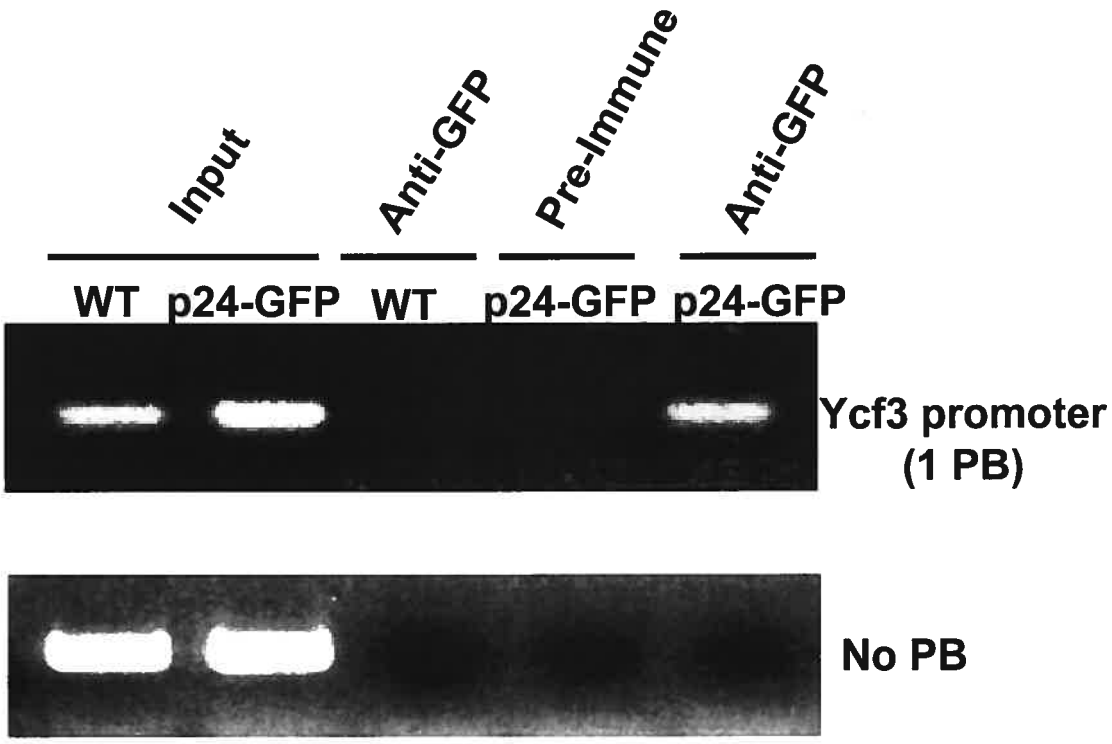

Input
Anti-GFP
Pre-Immune
Anti-GFP
WT
p24-GFP
WT
p24-GFP
p24-GFP
Ycf3 promoter
(1 PB)
No PB

Figure 3.13

Transgenic plants have altered p24 levels. Protein gel blot of wild-type, p24 overexpressing (S1 and S2) and p24 antisense (AS) potato plants. p24 antibodies were used. The bottom panel shows the same blot stained with Ponceau red.

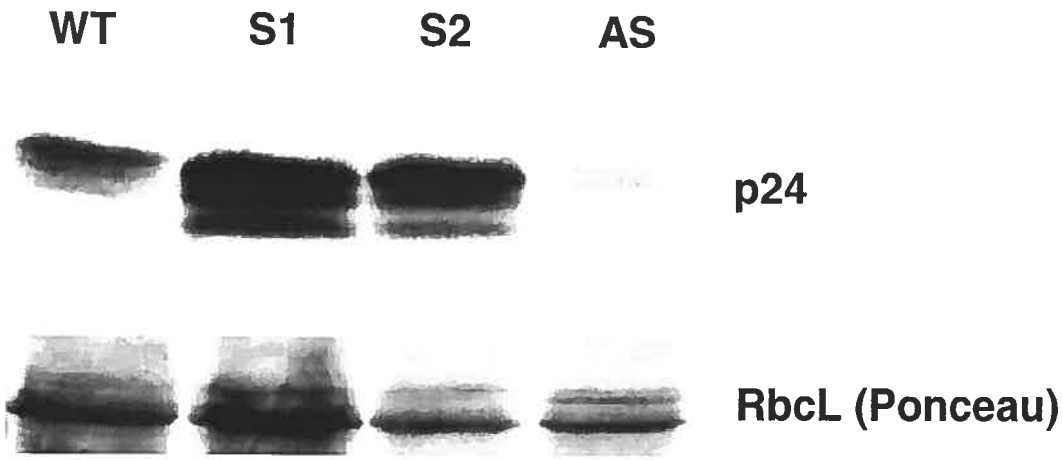
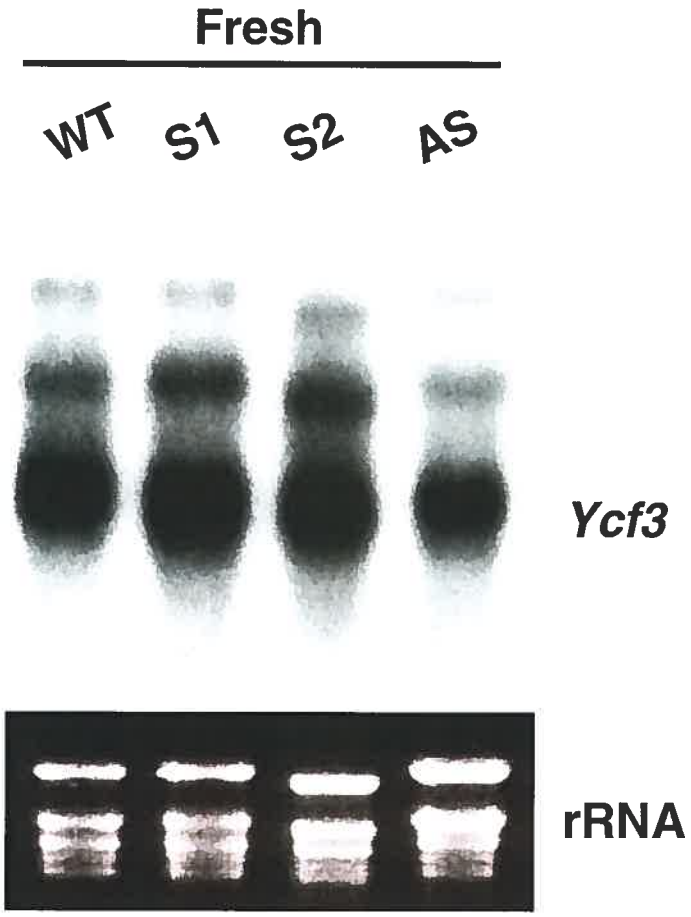

WT
S1
S2
AS
p24
RbcL (Ponceau)

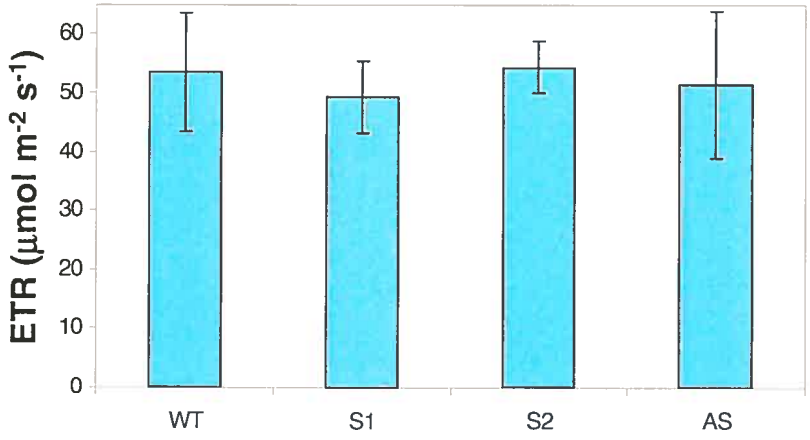
Figure 3.14

Ycf3 gene expression and photosynthesis in fresh potato leaves. **A** RNA gel blot of WT and p24 transgenic plants with a 1982 bp *Ycf3* probe. Two overexpressing (S1 and S2) lines and one underexpressing (AS) line were examined. Each lane contained 20 μg of total RNA. Ethidium bromide staining of ribosomal RNAs is shown as a loading control. **B** Electron transport rate (ETR) measured in WT and p24 transgenic lines. Results are averages (±SD) of 6 readings from two separate experiments. The results are not statistically significant ($p>0.486$).

A


Fresh
WT
S1
S2
AS
Ycf3
rRNA

B


ETR (µmol m-2 s-1)
60
50
40
30
20
10
0
WT
S1
S2
AS

is alternatively spliced into multiple transcripts and processing intermediates [40].

Previous results have shown that p24 proteins from potato and *Arabidopsis* are involved in stress induction of nuclear genes [6, 41]. To see if the control of chloroplast gene expression by p24 is also stress-dependent, we examined *Ycf3* RNA abundance in wounded potato plants. Figure 3.15a shows that *Ycf3* RNA levels were indeed greatly reduced in leaves of p24 antisense plants after wounding. Furthermore, the sense plants showed a slight increase in *Ycf3* expression under these conditions. This increase in Ycf3 expression was also observed with sense p24-GFP tobacco plants (data not shown). These results indicate that p24 has a major contribution in the expression of *Ycf3* after wounding and suggests that the role of p24 in stress-induced gene regulation is similar in chloroplasts and nuclei, providing a possible way by which both compartments could respond to the same stimuli.

It should be noted that, in the DNA immunoprecipitation experiment, the chloroplast isolation protocol required the leaves to be cut and incubated in the cold room overnight [37]. The results presented in Figure 3.11 therefore showed that p24-GFP is bound to chloroplast DNA of wounded leaves. It would be interesting to repeat the same experiment with fresh leaves to see if chloroplast p24 is free from the DNA in fresh tissues, as is p24 in the nucleus. If that is the case however, it would mean that p24 is present in nucleoids of fresh tissue chloroplasts without being bound to DNA (see Figure 3.5).

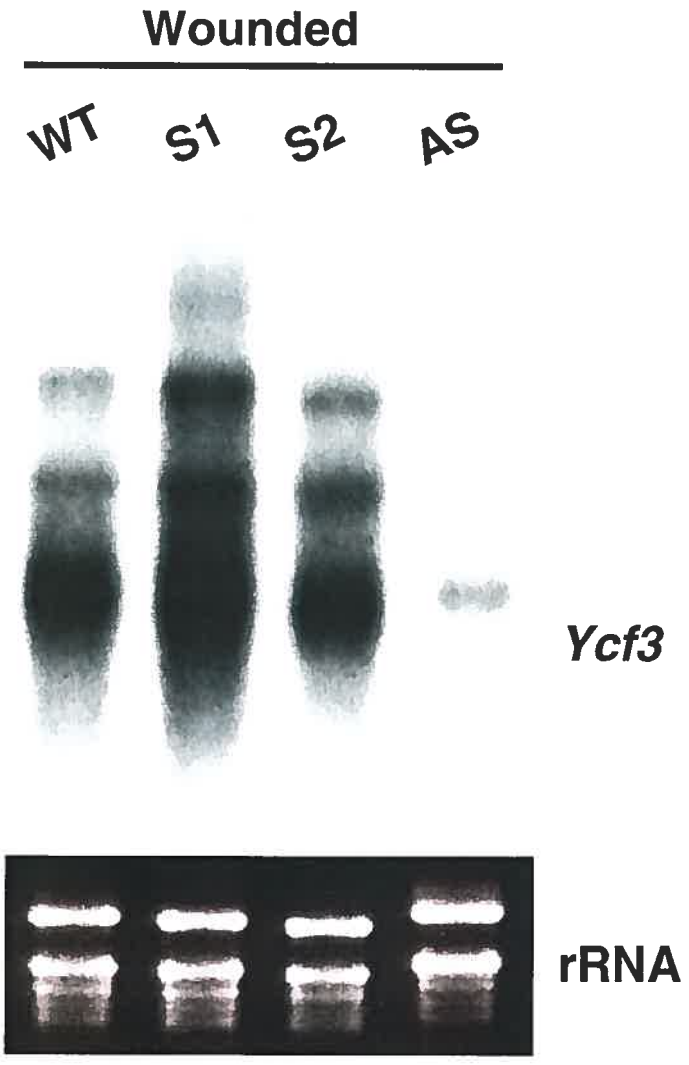
p24 could be involved in NEP-dependent transcription

Interestingly, the *Ycf3* gene has two active promoters. The first one directs transcription by a plastid-encoded polymerase (PEP), while the second controls a nuclear-encoded, phage-like polymerase (NEP). The PEP polymerase recognizes typical -35/-10 promoters and its DNA sequence specificity is provided by nuclear-encoded proteins similar to bacterial sigma factors. So far, six sigma factor genes have been found in the Arabidopsis nuclear genome [42-44]. The PEP polymerase plays an important role in chloroplast development and photosynthesis, as shown by mutant analysis [45-47]. These studies however also highlighted the importance of the NEP polymerase in plastid gene transcription. As a general rule, it was found that most photosystem I and II (PSI and PSII) genes are only transcribed by PEP,

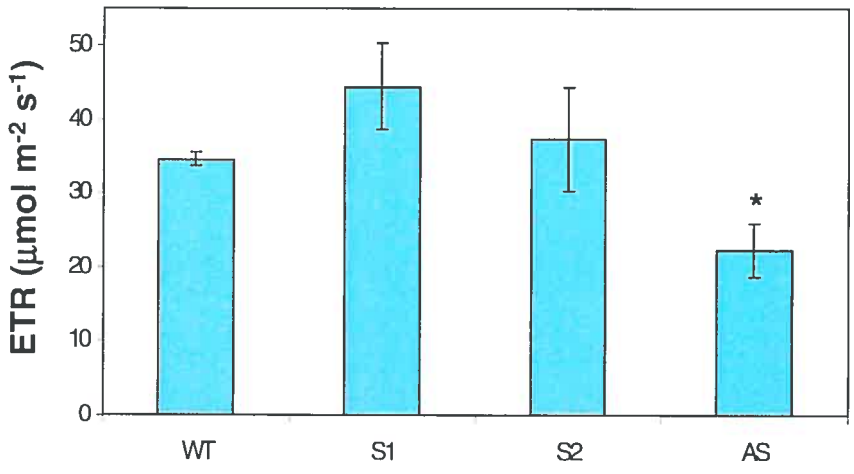
Figure 3.15

Ycf3 gene expression and photosynthesis in wounded potato leaves. **A** RNA gel blot of WT and p24 transgenic plants with a 1982 bp *Ycf3* probe. Two overexpressing (S1 and S2) lines and one underexpressing (AS) line were examined. Each lane contained 20 μg of total RNA. Ethidium bromide staining of ribosomal RNAs is shown as a loading control. **B** Electron transport rate (ETR) measured in WT and p24 transgenic lines. Results are averages (±SD) of 6 readings from two separate experiments. * Statistically significant compared to WT using a one-way ANOVA (p=0.001).

A


Wounded
WT
S1
S2
AS
Ycf3
rRNA

B


ETR (µmol m-2 s-1)
50
40
30
20
10
0
WT
S1
S2
AS
*

while most other genes have promoters for both polymerases. Finally some genes such as *accD* (encoding a subunit of acetyl-CoA carboxylase), the ribosomal protein genes *rpl33* and *rps18*, *ycf2* (encoding protein of unknown function) and the *rpoB* operon (encoding three PEP subunit genes) appear to be transcribed only by NEP [45-47]. Also, many of the NEP-transcribed mRNAs were more abundant in the PEP mutant plants than in the WT, suggesting an antagonistic relationship between the activities of both polymerases. It was hypothesized that NEP is most important in non-green plastids and in immature chloroplasts.

Recent studies, however, have shown using run-on transcription that most of the chloroplast genome is still transcribed in PEP mutants (albeit at different levels than in the WT plants) [48-49]. It appears that the differences in steady-state levels of chloroplast RNAs depend on post-transcriptional processing and stabilization/degradation, the rates of which depend on the specific polymerase used to transcribe each gene [48-50].

In the mustard *Ycf3* gene, the positions of the PEP and NEP promoters have been well-characterized [40] and the p24-binding site is adjacent to the NEP promoter, suggesting that p24 could facilitate NEP-dependent transcription. It should be noted however that not all chloroplast NEP promoters have an adjacent p24 binding site. p24 therefore likely controls a limited subset of NEP-dependent genes.

Stress-dependent control of photosynthesis by p24

The photosynthetic electron transport chain proceeds from the splitting of water at PSII to the electron carrier plastoquinone, the cytochrome b6/f complex and finally to PSI, where NADP+ is ultimately reduced through the action of ferredoxin/NADP oxidoreductase. Chlorophyll molecules capture light at each photosystem and excite the electrons, facilitating the reduction of downstream components. These photosynthetic oxidoreduction reactions create a proton gradient across the thylakoid membranes and the energy from this gradient is then harvested to produce ATP.

Ycf3 is essential for the assembly of the multi-subunit PSI [51]. Targeted inactivation of *Ycf3* results in a block in PSI accumulation and in fewer functional PSII centres. The latter is probably due to photooxidative damage caused by the lack of electron acceptors downstream of PSII [52] and/or to photosystem stochiometry adjustment, a mechanism by which the redox state

of the plastoquinone pool dictates the abundance of each photosystem as to maximize the efficiency of photosynthesis [12].

Because p24 affects *Ycf3* gene expression in a wound-dependent fashion, we expected that photosynthesis would be altered in the wounded p24 transgenic plants, but not in their fresh tissues. This was measured by determining the electron transport rate (ETR), which is indicative of the amount of electrons passing through PSII during steady-state photosynthesis. As expected, the fresh tissues of wild-type and transgenic potato plants had a similar ETR (Figure 3.14b). The wounded (see methods) p24 antisense leaves, however, had a lower ETR than the wild-type while the wounded sense tissues showed a slightly higher ETR, in agreement with their respective *Ycf3* expression levels (Figure 3.15b). These combined results indicate that p24 controls wound-dependent *Ycf3* gene expression and that this is correlated with wound-dependent changes in photosynthesis.

The role of p24 in photosynthesis during the wound response was further highlighted by incubation of leaf discs with a low concentration (10μM) of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU inhibits electron flow downstream of PSII and therefore causes over-reduction of PSII centres. DCMU had little effect on *Ycf3* expression (Figure 3.16a), compared to wounding without DCMU. However, it had a major impact on the ETR, leading to a 92% reduction in the antisense plants (Figure 3.16b). In contrast, the same concentration of DCMU had no significant effect on ETR in the sense plants, suggesting that the higher level of *Ycf3* expression in these plants compensates for the effect of DCMU (Figure 3.16b). It should be noted that the levels of *Ycf3* expression presented in Figures 3.14a, 3.15a and 3.16a can be compared as they are from the same RNA gel blot.

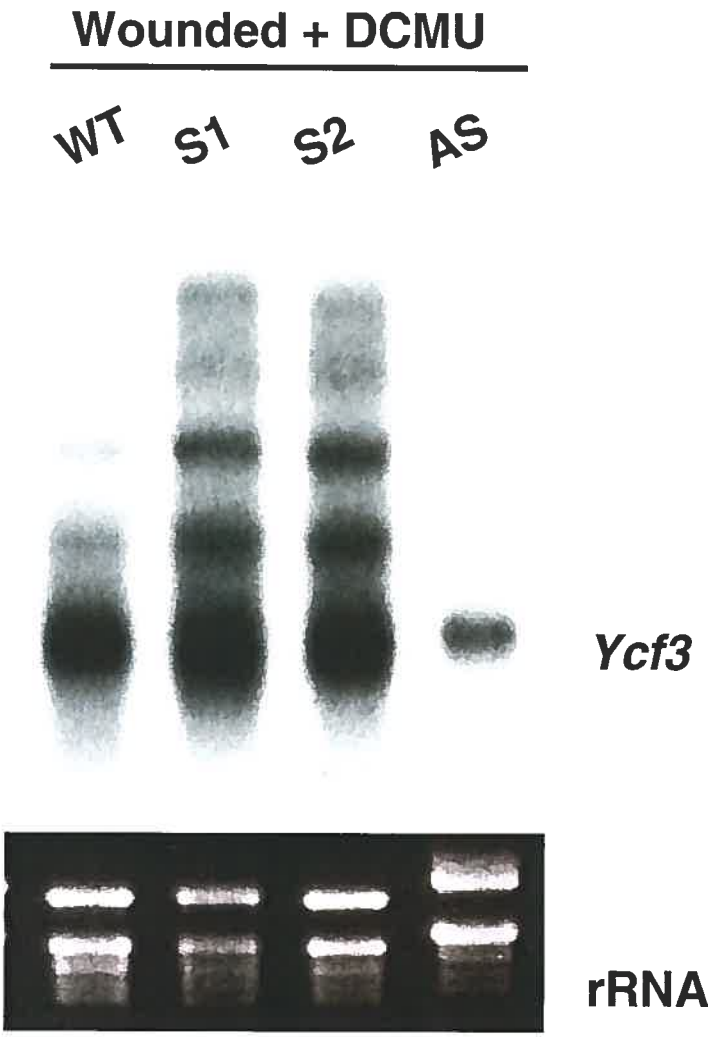
Role of cyclic electron flow in stress defence

During stresses such as wounding, treatment with a pathogen elicitor or ozone, drought and anaerobiosis, linear photosynthetic electron flow is down-regulated [53-57]. The mechanism is not known but is thought to involve feedback control of photosynthesis by the altered ATP/ADP and NADPH/NADP+ balances in stressed leaf cells [58]. Mechanisms to dissipate excess light energy therefore need to be activated in part because the unused

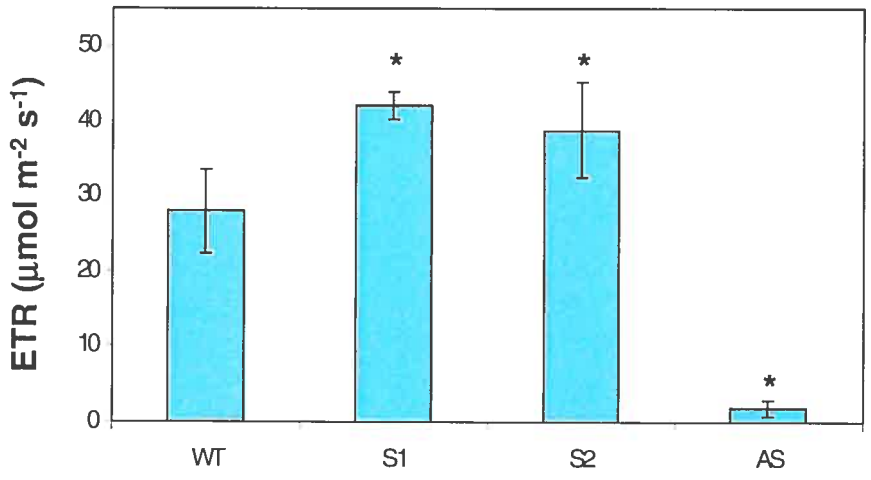
Figure 3.16

Ycf3 gene expression and photosynthesis in wounded potato leaves treated with DCMU. **A** RNA gel blot of WT and p24 transgenic plants with a 1982 bp *Ycf3* probe. Two overexpressing (S1 and S2) lines and one underexpressing (AS) line were examined. Each lane contained 20 μg of total RNA. Ethidium bromide staining of ribosomal RNAs is shown as a loading control. **B** Electron transport rate (ETR) measured in WT and p24 transgenic lines. Results are averages (±SD) of 6 readings from two separate experiments. * Statistically significant compared to WT using a one-way ANOVA (S1: $p=0.001$, S2: $p=0.04$, AS: $p<0.001$).

A


Wounded + DCMU
WT
S1
S2
AS
Ycf3
rRNA

B


ETR (µmol m-2 s-1)
50
40
30
20
10
0
*
*
*
WT
S1
S2
AS

light energy in PSII poses the threat of superoxide formation following oxidation of molecular oxygen by triplet electrons in the PSII chlorophyll antennas. PSI plays a central role under these conditions as a downstream electron acceptor for PSII and as a participant in cyclic electron flow. It has been shown that there is a switch from linear to cyclic electron flow in response to stress and that this has a protective role [55].

Cyclic electron flow around PSI scavenges electrons and helps to maintain the proton gradient across thylakoid membranes [59]. This is necessary to maintain appropriate levels of ATP in the cell, and for the function of other light dissipation mechanisms such as non-photochemical quenching (NPQ) [60]. NPQ is a process that normally dissipates the extra energy as heat and is caused in part by the de-epoxidation of the pigment violaxanthin into the light-quenching molecule zeaxanthin [61]. NPQ requires a strong proton gradient across the thylakoid membranes to function [61].

One possible explanation for the switch to occur between linear and cyclic electron flow could be that photosynthetic gene expression (ie. by the PEP polymerase) is down-regulated and that gene expression involved in cyclic electron flow and homeostasis (ie. by the NEP polymerase) is up-regulated. We are hypothesizing that this occurs, at least in the *Ycf3* promoter. This would be a long term adaptive response however and faster mechanisms, such as state transitions (a movement of antenna molecules from PSII to PSI), occur at the onset of the stress [62]. Interestingly, in WT plants, *Ycf3* gene expression does not change from fresh to wounded leaf tissues (Figures 3.14a and 3.15a). However, wounding affects *Ycf3* in p24 antisense plants. These results show that, in WT plants, a p24-dependent gene expression mechanism is activated in response to stress and this prevents the down-regulation of *Ycf3* under these conditions. As described before, wounding can lead to photooxidative stress. In this context, the maintenance of *Ycf3* expression and photosynthetic electron transport by p24 during the wound response will likely have a protective effect.

Under normal conditions, the gene *PsaA* that is downstream of *Ycf3* is expressed via its own PEP promoter [40]. However, in PEP mutant plants, larger RNAs containing both *PsaA* and *Ycf3* are up-regulated, most likely transcribed from the NEP promoter upstream of *Ycf3* [49]. Interestingly, larger *Ycf3* RNAs are particularly affected in wounded p24 transgenic plants,

compared to WT plants (Figure 3.15a). This result supports the ideas that 1) p24 contributes to NEP-dependent gene expression and that 2) there is a competition between NEP- and PEP-dependent transcription mechanisms.

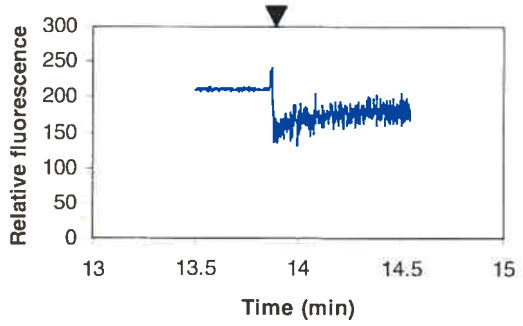
p24 antisense plants are defective in chlororespiration

One form of cyclic electron flow is chlororespiration, which is due to the cycling of electrons from reduced donors in the chloroplast stroma, such as NADH, back to plastoquinone [63]. Chlororespiration is accomplished by the NAD(P)H-dehydrogenase (NDH) complex, which is composed of 11 to 16 subunits [64]. In practice, chlororespiration can be visualized as the recovery of chlorophyll fluorescence in the dark, after illumination with high light [65]. This transient increase in fluorescence is due to the reduction of the plastoquinone pool that leads to closure of PSII reaction centers [66]. The change in fluorescence is typically small and any difference is likely to be significant. Interestingly, the recovery of chlorophyll fluorescence was impaired in the potato p24 antisense plants, compared to the WT plants (Figure 3.17). This lack of fluorescence recovery in p24 antisense plants is reminiscent of *ndhB* mutant plants that are deficient in cyclic electron transport around PSI [67]. In contrast, the potato p24 overexpressing plants displayed a slightly more important fluorescence recovery than the WT. These results are consistent with the effect of p24 on *Ycf3*. If PSI is not assembled correctly, because of a lack of *Ycf3*, it is likely that less cycling electron flow will occur. However, it is possible that these observations are also due to a direct effect of p24 on *ndh* genes. The p24 binding site can be found inside the first and last exons of *ndhA*, as well as inside the *ndhF* gene (Table I). These can be internal transcription regulation sites, or may be responsible for post-transcriptional processing. As a single-stranded nucleic acid binding protein, p24, like the *PR-10a* repressor SEBF, could control RNA metabolism in addition to transcription. The involvement of specific transcription factors such as p24 in transcription and post-transcription could explain the observed polymerase-dependent differences in chloroplast RNA metabolism [48-50].

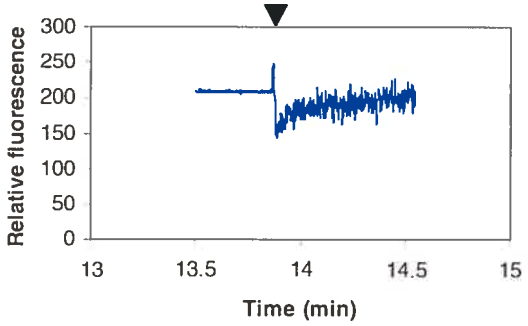
It is likely that the expression of PSI and NDH genes is coordinated during stress, in order to favour cyclic electron flow. The phenotype of p24

Figure 3.17

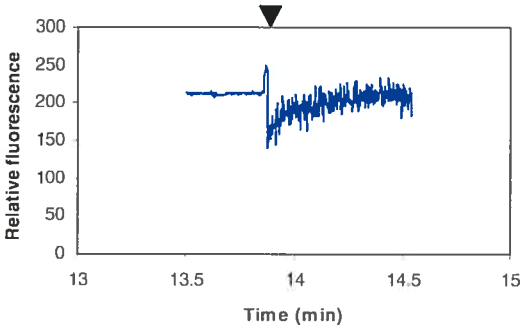
Steady-state chlorophyll fluorescence analysis of wounded WT and p24 transgenic leaf tissues. In the dark, following illumination, a transient increase in fluorescence due to cyclic electron flow occurs in WT, sense, but not antisense plants. In addition, the fluorescence recovery is more important in sense plants than in WT and antisense plants, suggesting that more cyclic electron transport occurs in plants overexpressing p24. Closed triangle: actinic light off. The increase in fluorescence occurs after the actinic light is turned off.


Relative fluorescence
300
250
200
150
100
50
0
13
13.5
14
14.5
15
Time (min)

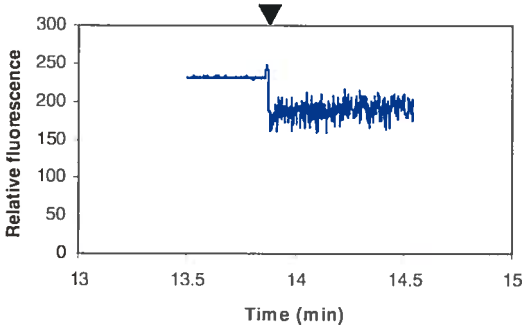
WT


Relative fluorescence
300
250
200
150
100
50
0
13
13.5
14
14.5
15
Time (min)

S1


Relative fluorescence
300
250
200
150
100
50
0
13
13.5
14
14.5
15
Time (min)

S2


Relative fluorescence
300
250
200
150
100
50
0
13
13.5
14
14.5
15
Time (min)

AS

transgenic plants is therefore probably due to a combination of the action of p24 on both PSI and NDH (and other genes involved in related processes).

p24 antisense plants lack the M transition

As mentioned above, cyclic electron flow serves in part to maintain the proton gradient across thylakoid membranes. Accordingly, the potato p24 antisense plants also lack the "M transition" during the initial decrease in fluorescence (Figure 3.18). The M transition is suggested to be linked to the establishment of the proton gradient [68] and this suggests that p24 antisense plants have a reduced proton gradient across chloroplast thylakoid membranes. Interestingly, mutant plants for the *PGR5* gene involved in cyclic electron flow display a reduced proton gradient, possibly due to increased conductivity of the ATP synthase, in addition to decreased linear electron flow [69].

Physical separation of PSII and PSI

PSII is present mainly in the stacked thylakoid membranes, while PSI is present in both the unstacked thylakoids and at the periphery of stacked thylakoids [70]. It has been suggested that the physical separation of the photosystem pools allows for fine tuning of the balance between linear and cyclic electron flow in the chloroplast [70]. Accordingly, functional studies of photosynthesis have suggested that there are two distinct pools of PSI and that, in response to stress, up to 30% of all PSI centres become involved in cyclic electron flow [58].

If during stress, when cyclic electron flow is favoured, the newly synthesized PSI is inserted in a "cyclic electron flow" pool, then it is possible that, by controlling *Ycf3* (and therefore PSI) at specific times (i.e. during stress), p24 contributes mainly to cyclic electron flow. Under normal conditions, other transcription factors would maintain steady-state *Ycf3* expression (ie. through the PEP promoter). This would explain the chlororespiration and M transition results described above. Alternatively, or in addition to this possibility, p24 may control other genes involved in cyclic electron flow.

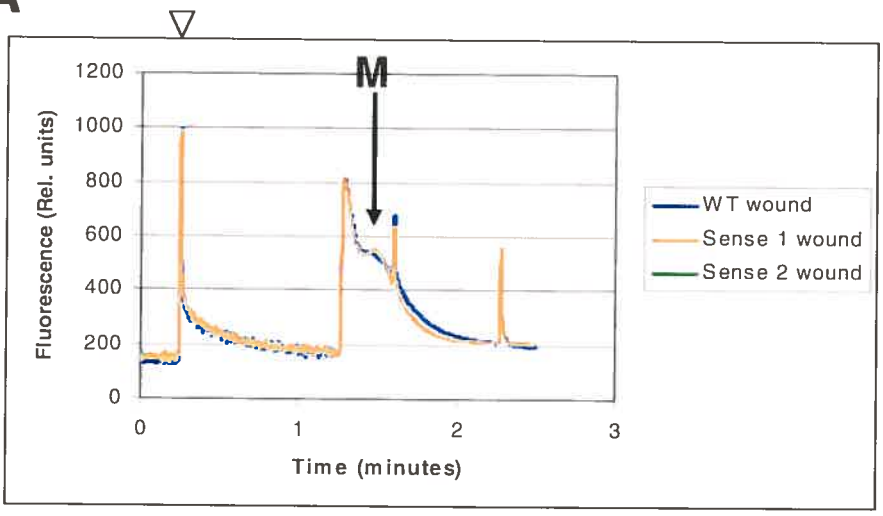
Photosynthesis-dependent nuclear gene expression

As photosynthetic signals affect nuclear gene expression [12], changes in *Ycf3* levels and photosynthesis in *p24* transgenic plants should be reflected at the nuclear level. We therefore determined whether the expression of

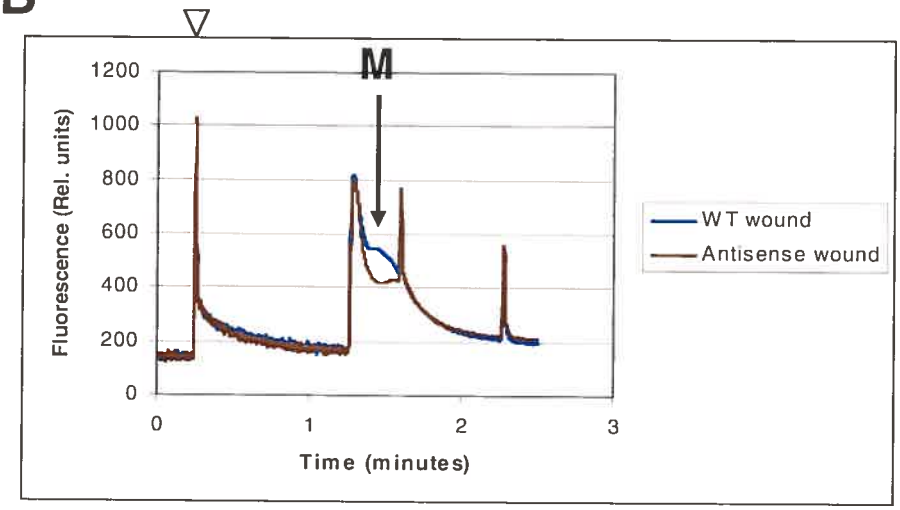
Figure 3.18

Early fluorescence events. Following fluorescence induction upon actinic light illumination, the decrease in fluorescence is transiently delayed (M transition). This secondary peak in fluorescence is present in leaf tissues from wounded WT and p24 Sense plants (A) but not in antisense plants (B), suggesting that p24 underexpressing plants do not support a strong proton gradient across the thylakoid membranes. Open triangle: Saturating light flash.

A


M
1200
1000
800
600
400
200
0
Fluorescence (Rel. units)
0
1
2
3
Time (minutes)
WT wound
Sense 1 wound
Sense 2 wound

B


M
1200
1000
800
600
400
200
0
Fluorescence (Rel. units)
0
1
2
3
Time (minutes)
WT wound
Antisense wound

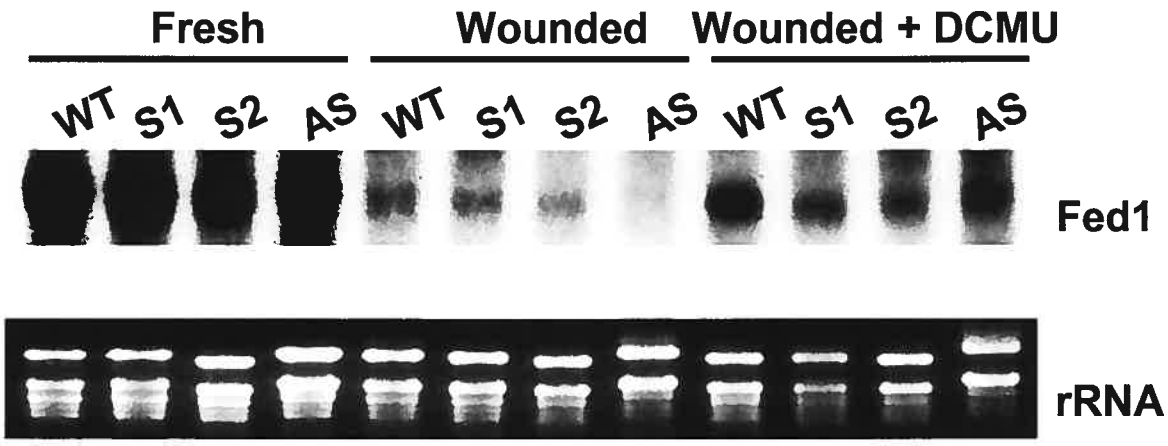
Fed1, a nuclear gene encoding ferredoxin, is changed in *p24* transgenic plants. We selected this gene because it is positively regulated by photosynthetic electron flow (PEF) [70] and is unlikely to be controlled by nuclear p24, as it contains no p24 binding site. While *Fed1* expression was not different in WT and *p24* potato transgenic fresh leaves, it was greatly reduced after wounding (Figure 3.19). This reduction in *Fed1* expression was more pronounced in antisense *p24* plants.

The repression of *Fed1* expression observed in the antisense plants was rescued by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron flow, confirming that this repression is regulated by photosynthesis (Figure 3.19). Interestingly, in fresh tissues, DCMU is an inhibitor of *Fed1* expression [70], whereas we have found that it actually rescued *Fed1* expression to wild-type levels in wounded p24 antisense plants. This suggests that, under stress, the reduced flow of electrons to PSI from PSII, because of DCMU, allows the reducing potential of PSI to be better matched to the decreased demands of carbon fixation. This would likely reduce the importance of cyclic electron flow relative to linear electron flow and should theoretically lead to an accumulation of reduced photosynthetic products, mimicking a "normal" situation. Of note, the low concentration of DCMU used here did not inhibit linear electron flow completely.

This could also explain why overexpressing plants are not rescued by DCMU to the same extent, because the increased *Ycf3* (and PSI activity) in these plants maintains the imbalance between PSII and PSI activity. In contrast, DCMU had little effect on the modified expression of *Ycf3* in wounded transgenic plants (Figure 3.16a). This suggests that p24 controls *Ycf3* independently of PEF, in agreement with our DNA immunoprecipitation results (Figure 3.12). We conclude that, under stress, the chloroplast p24 protein generates a photosynthetic signal that is perceived outside the chloroplast, resulting in altered nuclear gene expression.

Figure 3.19

Fed1 gene expression. RNA gel blot as in Figure 3.14 – 3.16, with a 485 bp *Fed1* probe. Each lane contained 20µg of total RNA.


Fresh
Wounded
Wounded + DCMU
WT
S1
S2
AS
WT
S1
S2
AS
WT
S1
S2
AS
Fed1
rRNA

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Chapter IV

Intra-cellular dynamics of p24

Introduction

The previous chapter has demonstrated the dual chloroplast/nucleus localization of the p24 transcriptional activator. Moreover, it appears that, in both chloroplasts and nuclei, p24 activates transcription in a stress-dependent manner. The dual localization of p24 therefore represents a possible mechanism by which different cell compartments could respond to the same stimuli.

One important issue that remains unresolved however is the mechanism by which p24 localizes to both compartments. Most nuclear encoded chloroplast-localized proteins possess a N-terminal transit peptide and are translocated across the chloroplast double-membrane via channels called "translocon at the outer envelope membrane of chloroplasts (Toc)" and "translocon at the inner envelope membrane of chloroplasts (Tic)" [1]. Protein translocation occurs at sites where the two membranes are held in close proximity [2]. Following import, the transit peptide is cleaved by the processing peptidase in the stroma (SPP), which is a metalloendopeptidase related to the β subunit of the mitochondrial processing peptidase [3]. p24 appears to go through this import pathway as it possesses a transit peptide and is processed to its mature form in vivo.

While chloroplast protein import occurs post-translationally, it is thought that pre-proteins are maintained in a partially unfolded state, as they come off the ribosome, by the chaperone Hsp70 [4]. Furthermore, many transit peptides possess phosphorylation sites that allow interaction with 14-3-3 proteins and the interaction of pre-proteins with Hsp70 and 14-3-3 factors is thought to serve as a guidance complex that increases import efficiency [5]. This theory is at least partially challenged by the recent finding that mutations of predicted phosphorylated residues in transit peptides did not alter import competence of preproteins [6]. Another model suggests that one protein from the Toc complex (Toc159) is present in a soluble form in the cytoplasm and directs the newly synthesized preproteins to the outer chloroplast membrane [7, 8]. This theory is also disputed however [9]. Nonetheless, it is very likely

that chloroplast import is tightly regulated, in order to avoid mistargeting to other organelles, and that cytoplasmic factors help to direct proteins to the chloroplast.

According to this model, proteins with chloroplast transit peptides (including p24) should localize exclusively to chloroplasts. It is known however that the chloroplast import machinery is developmentally regulated, with chloroplasts in older tissues progressively losing their ability to import proteins [10]. One example is starch phosphorylase that ceases to be imported into older potato tuber amyloplasts [11]. Interestingly, in that case, the cytosolic protein was found to have its transit peptide cleaved, suggesting that there is a cytosolic form of the SPP or that another processing site in the protein is used [11]. Another level of control is at the Tic complex, where components are regulated by the redox status inside the chloroplast, suggesting control of chloroplast protein import by light and bioenergetics in the stroma [12]. Furthermore, calcium also regulates the import of proteins containing a transit peptide [13]. This suggests that chloroplast import could be affected during conditions such as the defence response. In agreement with this hypothesis, the import of polyphenol oxidase by chloroplasts is enhanced by the defence signalling molecule jasmonic acid [14]. Similarly, abiotic stresses differentially affect the import of proteins into plant mitochondria [15].

It is therefore possible that, in certain tissues and under certain conditions, p24 is synthesized but not imported into chloroplasts. It would then accumulate in the cytoplasm, where it could eventually be imported into the nucleus. This is unlikely however, as the dual localization of p24 as been shown in a single cell, suggesting that both chloroplast and nuclear import of the p24 protein can function simultaneously. Furthermore, p24 does not possess a characteristic nuclear localization signal and, as it forms a tetramer, it would theoretically be too large to diffuse freely into the nucleus [16]. We cannot exclude the possibility however that p24 only tetramerizes in the nucleus.

Interestingly, examples exist that suggest a certain flexibility in the intracellular targeting of proteins. In addition to the dually localized proteins mentioned in the previous chapter, several plant proteins exhibit both chloroplast and mitochondrial localization [17]. It was found that this dual

targeting resulted from overlapping signals in the transit peptides of these proteins [18, 19]. In vitro however, mitochondria were found to be able to import purely chloroplastic proteins, whereas the opposite could not be achieved [20]. In an import assay containing both organelles, no mistargeting occurred [20]. This suggests that chloroplast import is more selective and that additional factors must be necessary in vivo to avoid inappropriate targeting to plant mitochondria. In contrast, a recent study showed developmentally regulated "mistargeting" of a protein carrying a vacuolar sorting signal to plastids of young sugarcane and Arabidopsis leaves [21]. Targeting signals therefore do not allow exclusive localization to a single compartments and it is probably the interaction of the signal and its cellular context that dictates the final localization(s) of a protein. In further support of this hypothesis, it was recently shown that RB60, a chloroplast protein disulfide isomerase (PDI) from the photosynthetic algae *Chlamydomonas reinhardtii*, also localizes to the endoplasmic reticulum (ER) [22]. In chloroplasts, RB60 is in the stroma as well as tightly bound to thylakoid membranes [23]. RB60 binds to and affects the translation of the chloroplast photosystem I *PsbA* mRNA in response to changes in the redox potential [24]. In the ER, RB60 most likely acts as a typical PDI [22].

RB60 possesses both a chloroplast transit peptide, as well as a C-terminal ER retention signal. The first 50 amino acids of the protein are sufficient for both localizations however [22]. This transit peptide is cleaved in the ER but not in the chloroplast [22]. The authors hypothesize that differential protein-protein interactions dictate the various functions of RB60 in both compartments. They also suggest that proteins in the cytoplasm compete for binding to the newly synthesized RB60 polypeptide and determine its ultimate localization. This also appears to be the case for animal proteins that are targeted to both the mitochondrial membrane and the ER. Several pro- and anti-apoptotic proteins such as Bax, Bak, Bid and Bcl-2 have indeed recently been shown to have this dual localization and to coordinate mitochondria-ER communication in response to apoptotic signals [24-28]. It is thought that these proteins regulate the release of calcium from the ER and the subsequent permeabilization of the mitochondrial membrane and release of cytochrome c [26-27, 29]. Interestingly, Bcl-2, Bax and Bid are also localized in the nuclear membrane [25, 28] and both Bcl-2 and Bax have been

found inside the nucleus [30]. A recent study showed that calcium signalling and crosstalk between mitochondria and the ER is also important for PCD in soybean cells [31]. Whether plant proteins play similar roles as Bcl-2, Bax and Bid is not known however. Finally, communication between the ER and mitochondria is probably facilitated by physical contacts that form between these two compartments, a process controlled by the protein PACS-2 in animals [32]. Upon apoptosis, PACS-2 also controls the translocation of Bid to mitochondria [32].

The functional and physical association of the chloroplast with the secretory system is therefore not surprising, nor is it new. Early microscopic observations had shown that the ER sometimes comes in close proximity of plastids [33, 34]. Moreover, chloroplast thylakoids and the ER can exchange membrane lipids [35, 36]. Other biosynthetic pathways, such as that of the plant hormones gibberellins, are also shared between chloroplasts and the ER [37].

Association of chloroplasts with the ER may even date back to the endosymbiotic event that gave rise to chloroplasts. It has been shown in animals that the ER fuses with the plasma membrane of macrophages at the onset of phagocytosis [38]. Some intracellular pathogens are able to stop the maturation of the phagolysosome and can survive inside these non-lytic ER/phagosome compartments [38]. These results showed that the ER can unexpectedly fuse with other compartments and suggested a way by which organisms could find a safe haven inside host cells. This could partially explain the early events of endosymbiosis.

Some unicellular algae possess "complex plastids" that are thought to have resulted from a secondary endosymbiosis event [39] and are surrounded by 3 or 4 membranes [40]. Interestingly, in some groups of plastids with 4 membranes, the outer membrane is continuous with the ER and is known as "chloroplast ER" [41]. Protein import into these plastids is therefore greatly complicated by the additional membrane barriers and is still not very well characterized. Preproteins targeted to complex plastids contain a N-terminal, hydrophobic, ER-targeting signal peptide followed by a chloroplast transit peptide rich in hydroxylated and positively charged amino acids [40]. Once attached to the ER, these proteins proceed to the plastids via vesicles of the secretory pathway [40].

The recently proposed "secretory transport hypothesis" suggests that, following the primary and secondary endosymbiotic events and the transfer of genes from the symbionts to the host nucleus, protein transport back to the symbionts occurred via the secretory pathway, using the host machinery [42]. These proteins would have acquired a signal peptide and the adjacent region would have evolved into a chloroplast transit peptide to ensure specificity of plastid targeting (ie. to avoid targeting to other organelles such as vacuoles and lysosomes) [42]. Later, as specific receptors of transit peptides evolved in the outer plastid membrane, the signal peptide was lost because it was no longer necessary [42]. This view is supported by the finding that Toc159 bears sequence homology to signal recognition particle receptors involved in targeting to the ER [43].

Finally, to further illustrate the versatility of plastid membranes and of organelle import/export pathways, stromal proteins have been found to be exported from chloroplasts of *C. reinhardtii* in vesicles that fused with lytic vacuoles [44]. A similar phenomenon has been observed in soybeans, where chloroplasts were found to secrete lipid/protein globules into the cytoplasm during senescence [45]. Membrane tubular extrusions containing stroma (and therefore called "stromules") also extend from plastids, sometimes linking two plastids together [46]. Stromules are able to actively transport proteins [46-48]. They are sometimes associated with the plasma membrane and they have been observed to go through invaginations in the nucleus [49].

In summary, chloroplast protein import must be tightly regulated to avoid mistargeting to other organelles. Yet there appears to be a wide range of methods to achieve this import in the plant kingdom. The cell apparently has many more channels and doors than previously thought. The secretory pathway from which chloroplast protein import probably evolved may still be used in some instances and protein transport may not always be unidirectional. In animal cells, proteins are also more promiscuous than was believed previously.

The present chapter presents the localization of p24 in plastid stromules. Unexpectedly, these p24-containing stromules co-localize with the ER. The ER is also found to encircle chloroplasts in some instances and this is reminiscent of algal chloroplast ER. It is not inconceivable that p24 gains access to the secretory pathway from chloroplasts and that this is how it

reaches the nucleus. In apoptotic human dopaminergic SH-SY5Y cells, glyceraldehydes-3-phosphate dehydrogenase uses the golgi apparatus to transit to the nucleus [50].

Materials and methods

Tobacco cell cultures

Square (~5 mm^2) leaf sections from wild-type, p24-GFP and CT-GFP tobacco plants were placed on solid NT1 medium (1x MS salts, 100 mg/L myo-inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D, 180 mg/L KH_2PO_4, 3% (w/v) sucrose, 8 g/L agar, pH 5.8) [51] to induce callus formation. Callus pieces were then transferred to 50 mL of liquid NT1 medium in 250 mL conical flasks and incubated with continuous shaking in a growth chamber with a 16 hr photoperiod. Cells were transferred to fresh medium every 2-3 weeks.

Induction of xylogenesis was performed as described [52]. In short, cultured cells were transferred to 50 mL of liquid xylogenesis medium [NT1 medium minus 2,4-D, plus 0.23 mg/L 6-benzylaminopurine (BAP, Sigma)] and incubated with shaking for 48 hrs.

Laser-scanning confocal microscopy

GFP fluorescence (500-530 nm) was visualised with a Leica DM IRB/E laser-scanning confocal microscope using a 488 nm laser excitation source. Chlorophyll autofluorescence was visualised at 650-700 nm. Hand-made thin sections of fresh leaves or roots from in vitro grown plants were placed in sterile water on a slide, under a cover slip sealed with nail varnish, and examined directly. Cells in culture were visualised by placing a drop of cell culture on the slide, covering with a cover slip, and sealing with nail varnish. For ER and golgi staining, cells in culture were incubated with 0.01 μg/mL Bodipy-brefeldin A (Bodipy-bfa, Molecular Probes) for 10-30 min at room temperature, washed with fresh culture medium, and examined right away. Bodipy-bfa fluorescence (570-600 nm) was visualised using a 568 nm laser excitation source. GFP and Bodipy-bfa fluorescence images were collected sequentially, and no fluorescence cross-talk was observed under our conditions (not shown). Pseudocoloring of the images, maximal projections, and image overlays were done using the Leica confocal software (LCS).

Disruption of ER membranes was achieved by treatment of tobacco cell cultures with 50 μM nordihydroguaiaretic acid (NDGA, Sigma) for 10-30 min.

Fluorescence recovery after photobleaching

The Leica DM IRB/E laser-scanning confocal microscope is not specifically designed for fluorescence recovery after photobleaching (FRAP) experiments. The Leica confocal software was therefore programmed to perform the following actions sequentially. First, four images were taken at intervals of 5 s to ensure that the GFP fluorescence was not affected at the laser intensity used. Second, a square area (it is not possible to select round areas) corresponding to the nucleus was selected and the laser intensity increased to maximum. This area was scanned 20 times, over a period of 1 min. Under our conditions, this resulted in a decrease of over 90% of the GFP fluorescence in the nucleus. Fluorescence recovery was then monitored by taking an image of the entire cell every 15 s for 600 s, yielding a total of 41 images.

Results and discussion

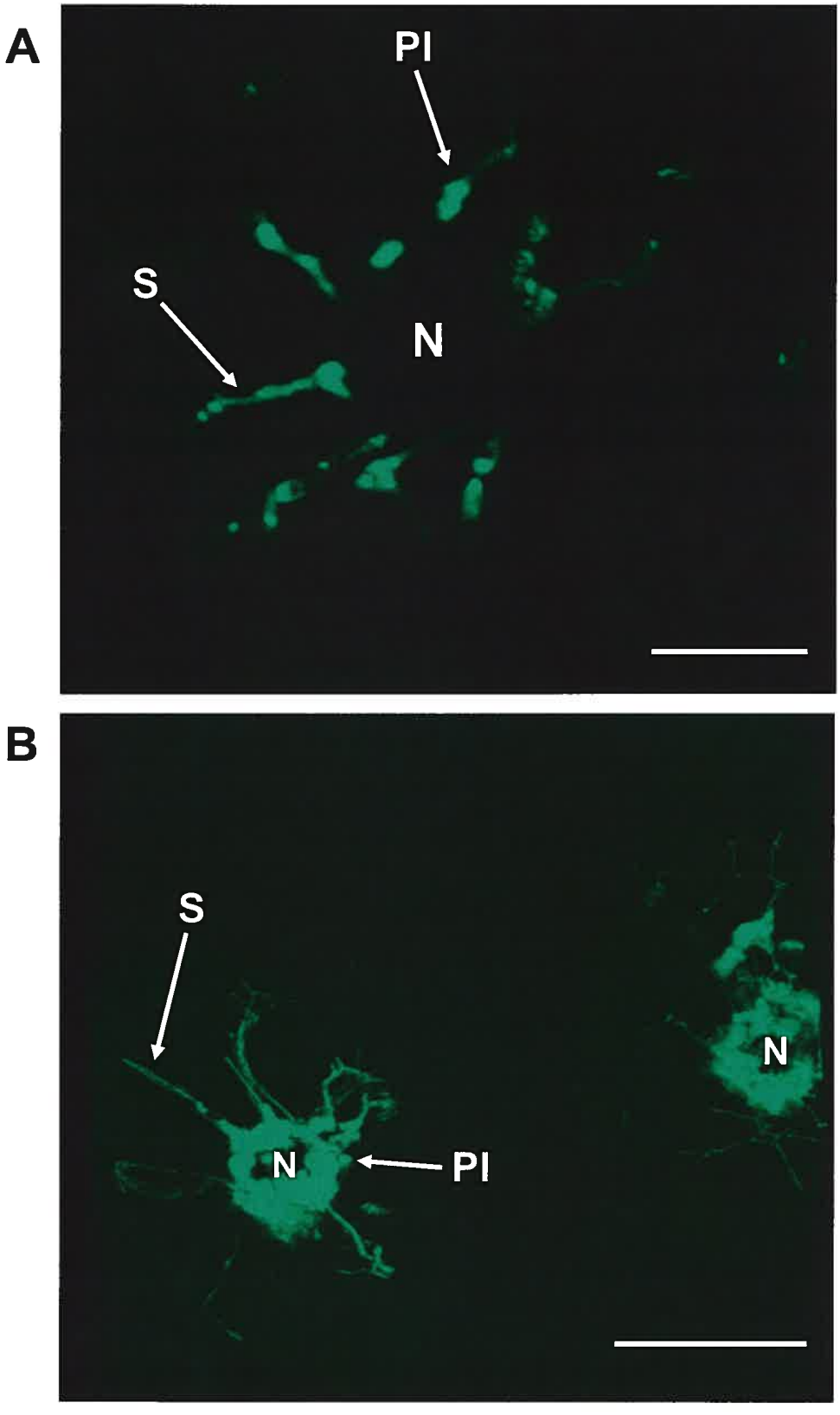
p24 is present in chloroplast extensions

The mechanism for dual localization of p24 is an open question. As seen previously, p24-containing plastids are often clustered around the nucleus. This provides the intriguing possibility that there could be a direct exchange of molecules between these two organelles, as has been suggested before [49, 51]. If this is the case, then which channel would p24 go through?

Plastids are known to sometimes have extensions, known as stromules because they are continuous with the stroma, that can extend to the plasma membrane and link plastids together [46-49, 51]. It has been shown that GFP can travel within stromules using what appears to be an active, ATP-dependent mechanism [46, 47]. Figure 4.1a shows p24-GFP fluorescence in root plastids and in stromules. It should be noted that root plastids are much smaller than leaf chloroplasts. This picture is virtually identical to those published with GFP alone, suggesting that p24-GFP is also

Figure 4.1

p24-GFP is found in plastid stromules. LSCM of root cells (A) and cells in culture (B) from transgenic p24-GFP tobacco plants. Maximal projections are shown. GFP fluorescence is pseudo-coloured in green. N = nuclei. Pl = plastids. S = stromules. Scale bars = 10.64µm (A) and 24.46µm (B).


A
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Pl

transported in stromules, though it remains to be determined whether p24-GFP is transported actively or moves by diffusion alone. Stromules are difficult to observe without GFP fluorescence, and it is therefore not possible to say whether some stromules do not contain p24.

In most root cells, p24-GFP is seen in stromules while, in leaf cells, p24-GFP stromules are very rarely seen. This is in accordance with the published fact that stromules are tissue-specific and developmentally regulated [51]. They occur more frequently in non-photosynthetic tissues, and very rarely in leaf cells. Our results therefore indicate that stromules containing p24-GFP appear in cells where p24 can be dually localized (ie. root cells). This begs the question as to whether stromules could be a direct channel to the nucleus.

p24 nuclear localization can be induced

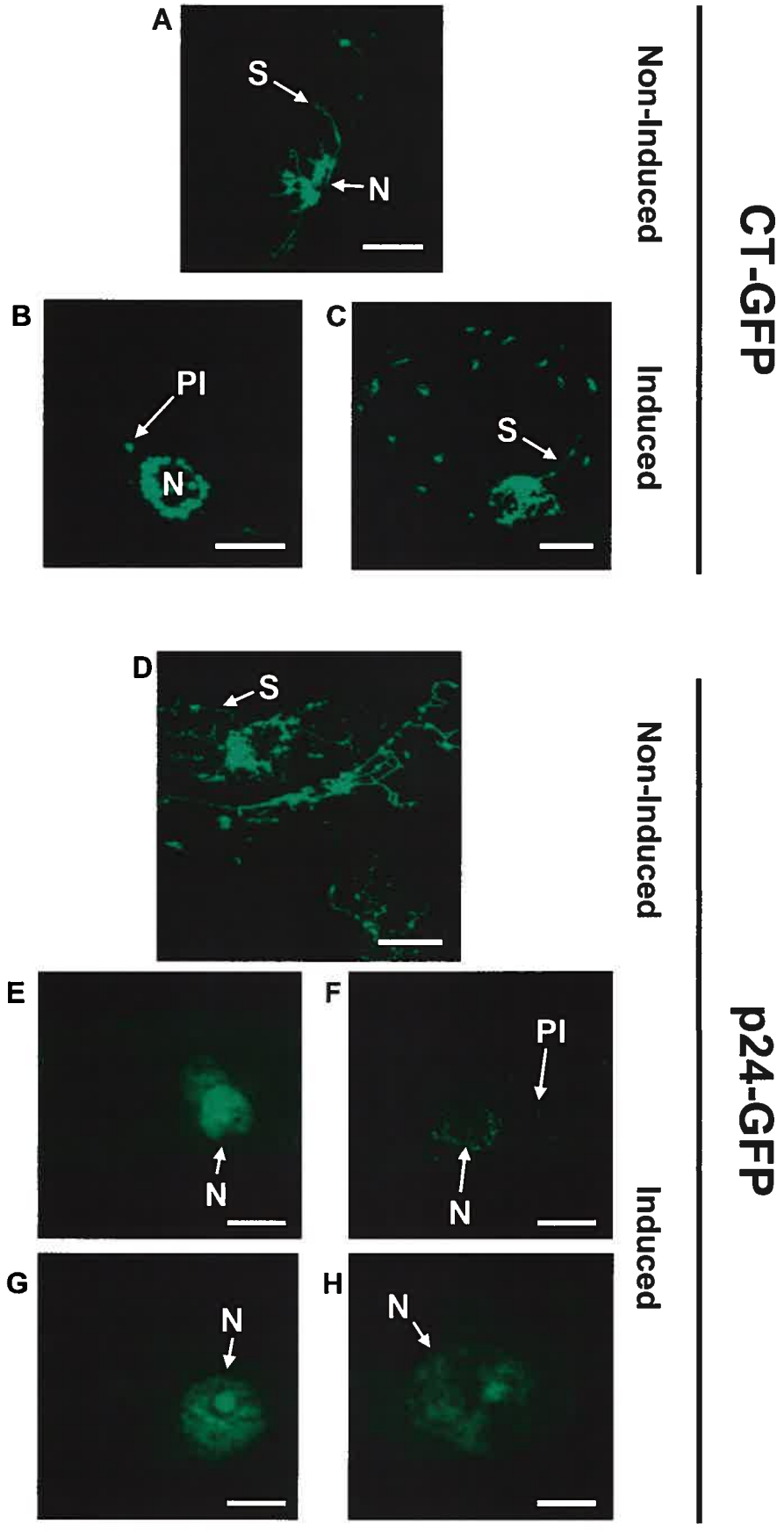
A cell culture system was developed to test this hypothesis. Plastids from cells in culture have very extensive stromules and are almost always clustered around the nucleus [51]. Transgenic p24-GFP cells also have extensive stromules (Figure 4.1b) but do not display dual localization of p24. Our cell cultures are derived from de-differentiated leaf cells. It appears that, though cell culture conditions stimulate the production of stromules, the stimulus for nuclear localization of p24 is missing.

In the nucleus, p24 is a transcriptional activator of *PR-10a*. Of note, in plant tissues, both wound- and pathogen-induced expression of *PR-10a* is strongest in the vasculature [53]. It was therefore hypothesized that the differentiation of cultured cells into vascular cells may induce the nuclear localization of p24. Published protocols allow the differentiation of culture cells into vascular tracheary elements (xylem). As xylem cells are dead cells, this differentiation ends in cell death.

We induced xylogenesis, by changing the hormonal balance in cell cultures, for a period of 48 hrs. This time point marks a peak in the expression of vascular gene markers, but precedes tracheary element formation which occurs 8-15 days post-induction [52]. Figure 4.2 shows that, in p24-GFP transgenic cells, dual localization of p24-GFP was induced by the xylogenesis treatment. Control CT-GFP cells however did not display any GFP fluorescence in the nucleus after induction. Of note, dual-localization of p24-GFP was not seen in all induced cells. Rather, it was seen in cells present in

Figure 4.2

p24-GFP accumulates in the nucleus when xylogenesis is induced. LSCM of CT-GFP and p24-GFP cells in culture from transgenic tobacco plants. The cells were examined before (non-induced) and after (induced) xylogenesis induction. GFP fluorescence is pseudo-coloured in green. Maximal projections are shown. N = nuclei. Pl = plastids. S = stromules. Scale bars = 20μm (A, B), 19.92μm (C), 20.35μm (D), 9.52μm (E), 14.84μm (F), 6.85μm (G), 4.47μm (H).


A
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Non-Induced
Induced
p24-GFP

large aggregates that appeared after induction. Interestingly, it was found that larger aggregates express larger quantities of vascular marker genes and are more likely to form tracheary elements [52]. This suggests that the dual localization of p24 is linked to the xylogenesis developmental program. Interestingly, stromules are also much less frequent in induced cells. Whether the stromules retract or fuse with other cellular membranes (ie. ER) remains to be determined. In any case, the possibility that the nuclear localization of p24 could be associated with a form of programmed cell death is reminiscent of the dual mitochondrial/nuclear localization observed with AIF, as described in the previous chapter.

Cytokinins as inducers of p24 nuclear localization?

To induce xylogenesis, the culture medium must be supplemented with the plant hormone cytokinin and deprived of auxin [52]. Notably, at the whole plant level, cytokinins were found to be responsible for vascular differentiation in the root [54]. Cytokinins have many other effects in plant cell cultures, however, and p24 dual localization could be a consequence of any of these outcomes. For example, cytokinins were found to induce nitric oxide (NO) formation in tobacco cell cultures [55] and NO is an important signalling molecule in the defence response [56]. Cytokinins were also found to induce cell division via a D-type cyclin in *Arabidopsis* cell cultures, but this effect also requires the presence of auxin [57]. Unsurprisingly, apoptosis is induced in *Arabidopsis* and carrot cell cultures by cytokinins [58]. In this study, cell death was observed after just 24 hours, but the hormone concentration used was 13-27 μM, which is much higher than the concentration used here (1 μM). Finally, the differentiation of plastids into starch-storing amyloplasts is stimulated by auxin depletion and by addition of cytokinins in tobacco BY-2 cell cultures [59, 60]. This is interesting, because both stromules and p24-dual localization are more frequent in non-photosynthetic, amyloplast-containing tissues.

The determination of the exact cause of p24 nuclear localization will require the study of each individual cytokinin effect. For example, it would be interesting to treat cell cultures with physiological concentrations of NO and determine whether p24 nuclear localization is induced.

Such experiments would help to determine "why" and "when" p24 is localized in the nucleus, but would not answer the question "how".

Nonetheless, the xylogenesis induction assay provides a system in which inhibitors of various protein transport processes can be assayed for their effect on p24 nuclear localization.

Close association of stromules with the secretory system

The secretory system is a possible candidate for p24 transport, given its close association with both plastids and the nucleus. Furthermore, in animals, at least two proteins (Bcl-2 and Bax) dually localized in mitochondria and the nucleus are also associated with the ER. NDGA is a compound that disrupts ER membranes but does not visibly affect the golgi [61]. Interestingly, preliminary results show that p24-GFP stromules completely disappear from tobacco cells in culture following NDGA treatment (Figure 4.3), suggesting that stromule membranes are either directly affected by NDGA or depend on ER membranes for their growth. Furthermore, stromules containing p24-GFP co-localize with the fluorescent ER marker Bodipy-Bfa (Figures 4.4 and 4.5). All observed stromules co-localized with ER, but not all ER membranes co-localized with stromules. Time-lapse imaging of WT tobacco cells in culture, stained with the same concentration of Bodipy-Bfa, showed the movement of ER strands and golgi vesicles (not shown), suggesting that the secretory pathway is not disrupted under these conditions. Bodipy-Bfa is known to stain ER and golgi membranes without any visible effect on the secretory pathway at low concentrations [62]. Negative effects on ER and golgi membranes observed at higher concentrations are delayed and are thought to result from the cleavage and release of Bfa by cellular enzymes, not from activity of Bodipy-Bfa per se [62].

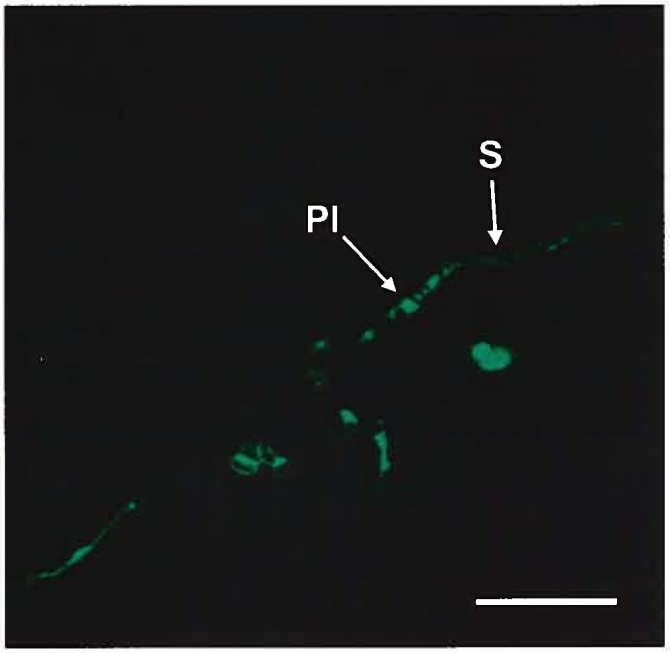
Interestingly, Figure 4.6 shows that membranes stained by Bodipy-Bfa not only co-localize with stromules, but also completely surround plastids in some instances. As can be seen on the graph, peaks of Bodipy-Bfa fluorescence are on either side of the wide GFP fluorescence peak, suggesting that ER membranes are surrounding the plastids containing p24-GFP. This is reminiscent of chloroplast ER observed in algae and suggests that, in some cells and under certain conditions, chloroplast ER may form in higher plants.

These results suggest a close association of stromules with the ER. Alternatively, stromule membranes may have ER-like attributes that allow staining by Bodipy-Bfa. In either case, it would now be interesting to test

Figure 4.3

p24-GFP stromules are sensitive to NDGA. LSCM of p24-GFP cell cultures from transgenic tobacco. The cells were either treated with the ER inhibitor NDGA or with an equivalent amount of DMSO. This experiment was performed twice. GFP fluorescence is pseudo-coloured in green. N = nuclei. Pl = plastids. S = stromules. Scale bars = 15.13µm (DMSO), 20µm (NDGA).

DMSO



NDGA

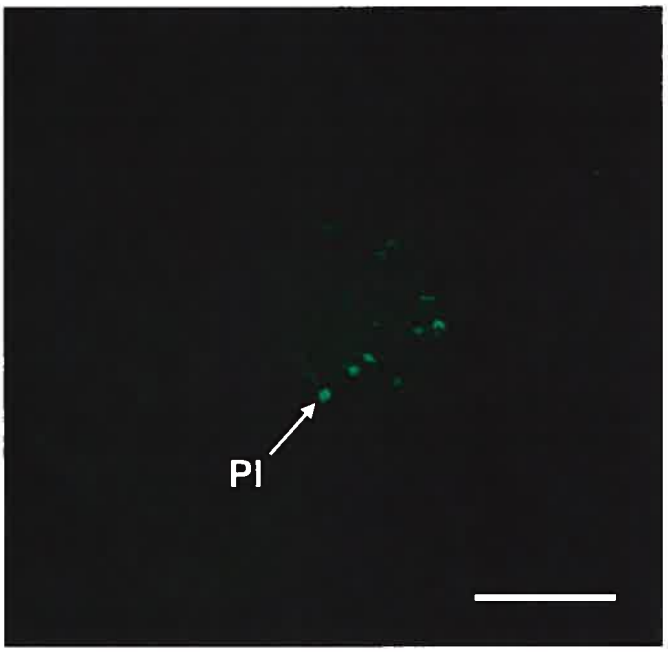
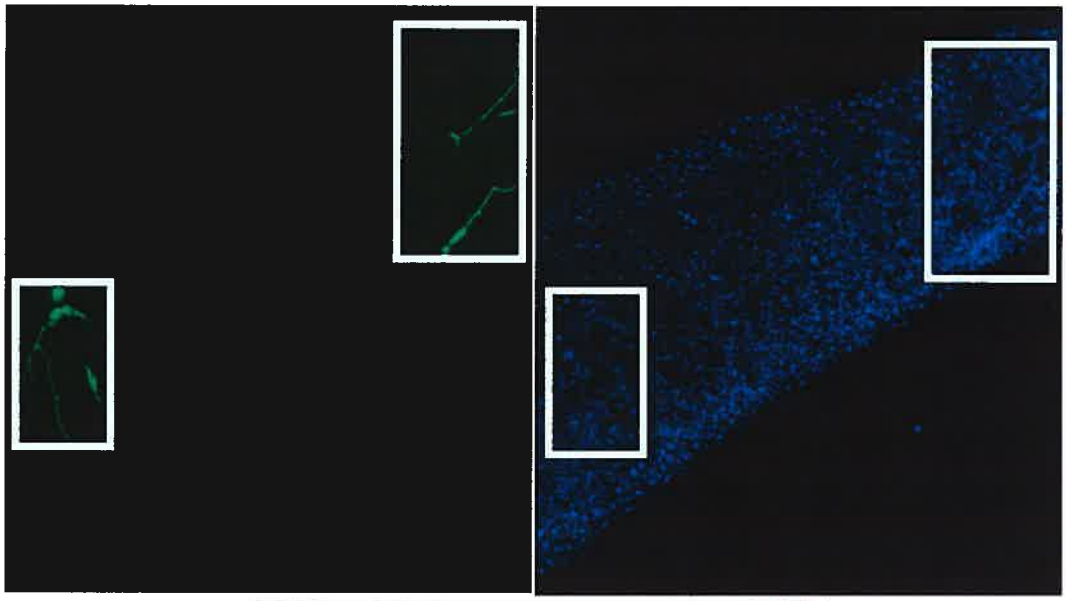


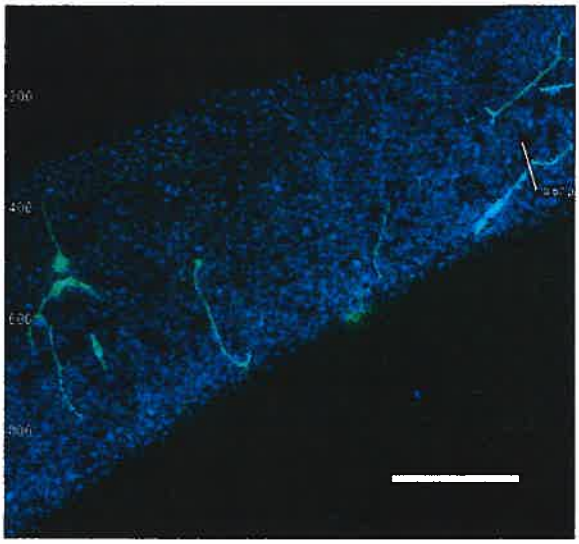
Figure 4.4

p24-GFP stromules co-localize with ER strands. LSCM of p24-GFP cell cultures from transgenic tobacco (only a part of a cell is shown), stained with the fluorescent ER marker Bodipy-Bfa. **A** The left panel shows GFP fluorescence, pseudo-coloured in green. The right panel shows Bodipy-Bfa fluorescence, pseudo-coloured in blue. Maximal projections are shown. Rectangles highlight areas of visible co-localization. **B** Overlay of the two images. A white line is drawn through an area of co-localization. Fluorescence intensity is measured along this line and plotted on the graph below for each fluorescent marker (Green = GFP, Blue = Bodipy-Bfa). Scale bar = 20μm.

A



B



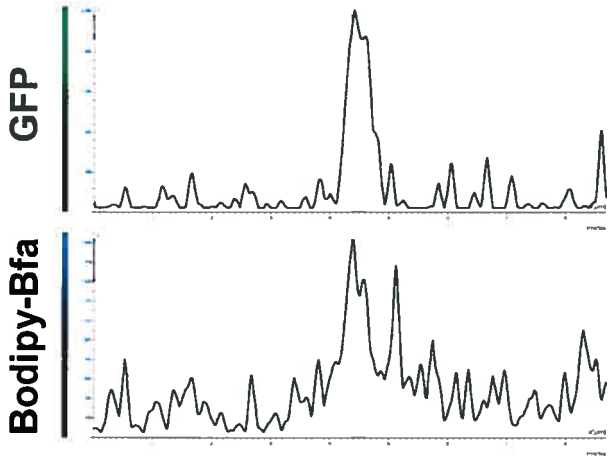
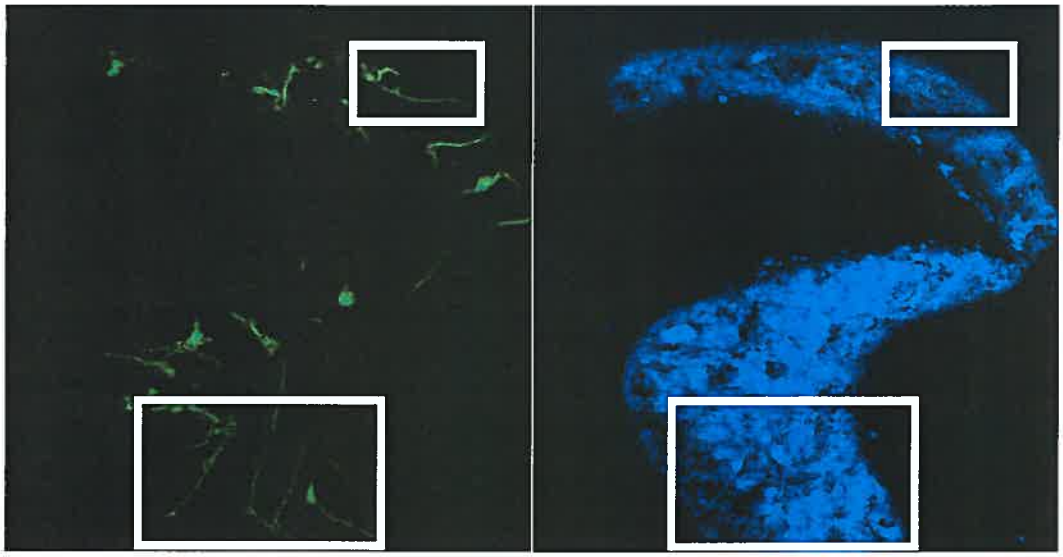


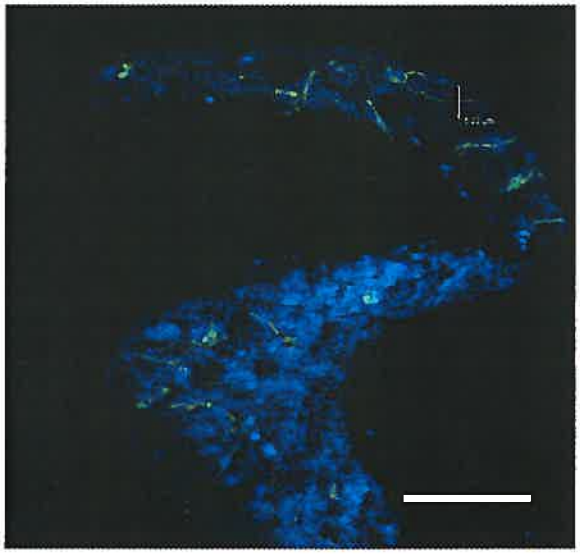
Figure 4.5

p24-GFP stromules co-localize with ER strands – Part 2. LSCM of p24-GFP cell cultures from transgenic tobacco (two cells are shown), stained with the fluorescent ER marker Bodipy-Bfa. **A** The left panel shows GFP fluorescence, pseudo-coloured in green. The right panel shows Bodipy-Bfa fluorescence, pseudo-coloured in blue. Maximal projections are shown. Rectangles highlight areas of visible co-localization. **B** Overlay of the two images. A white line is drawn through an area of co-localization. Fluorescence intensity is measured along this line and plotted on the graph below for each fluorescent marker (Green = GFP, Blue = Bodipy-Bfa). Scale bar = 20μm.

A



B



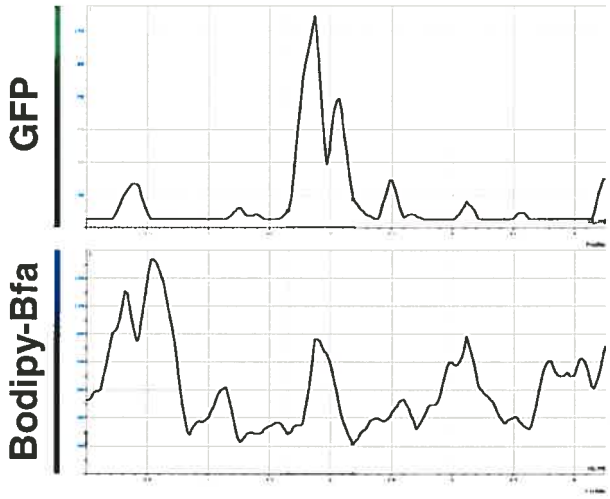
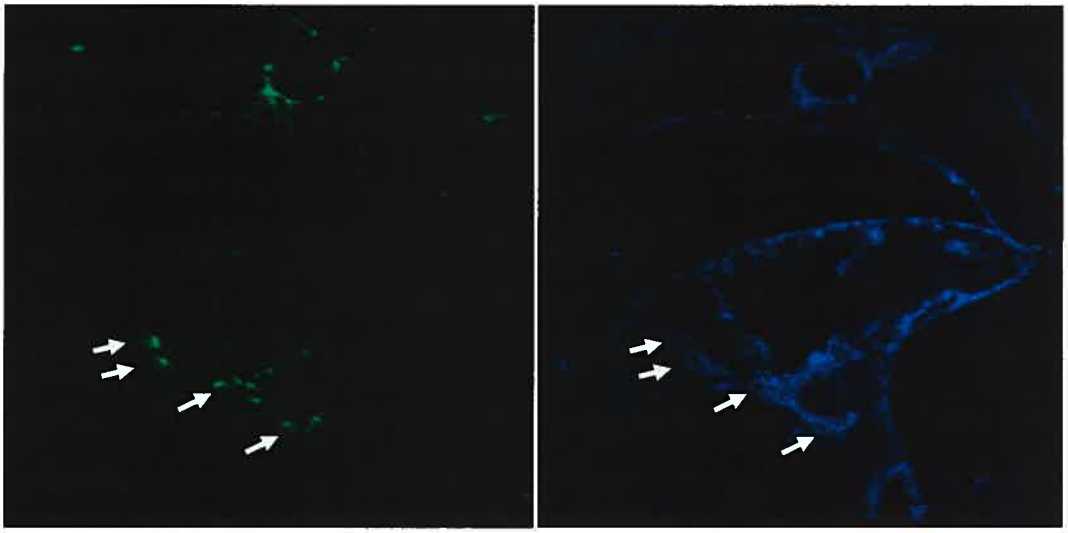


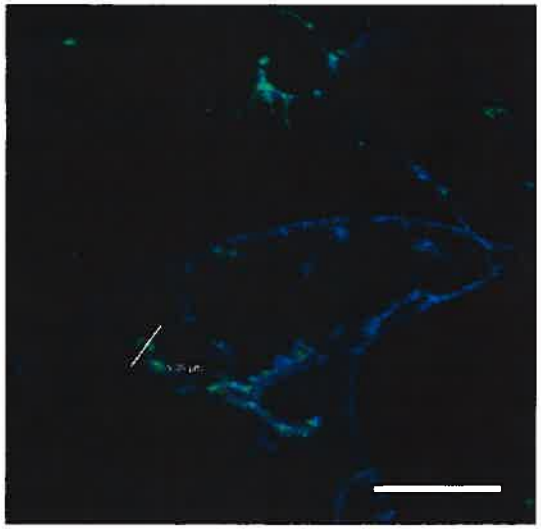
Figure 4.6

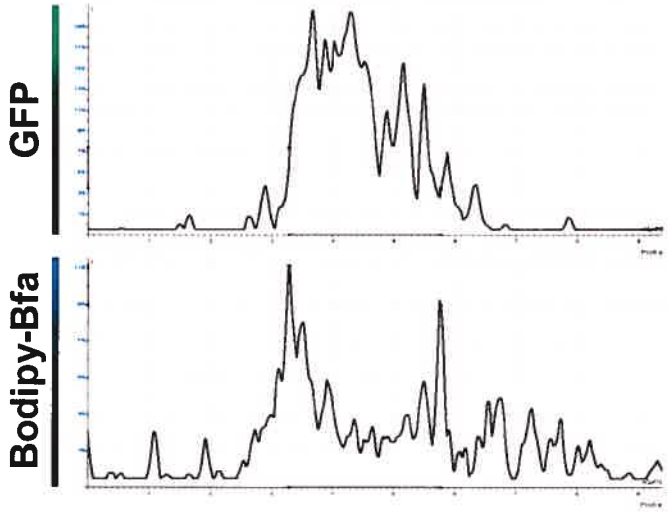
Tobacco cells have chloroplast ER. LSCM of p24-GFP cell cultures from transgenic tobacco (two cells are shown), stained with the fluorescent ER marker Bodipy-Bfa. **A** The left panel shows GFP fluorescence, pseudo-coloured in green. The right panel shows Bodipy-Bfa fluorescence, pseudo-coloured in blue. Maximal projections are shown. Arrows point to plastids surrounded by ER. **B** Overlay of the two images. A white line is drawn through a plastid surrounded by ER. Fluorescence intensity is measured along this line and plotted on the graph below for each fluorescent marker (Green = GFP, Blue = Bodipy-Bfa). Scale bar = 20μm.

A



B





whether inhibitors of the secretory pathway (such as NDGA) can inhibit p24 nuclear localization in the xylogenesis assay. However, such experiments will be complicated by the rapid action of NDGA compared to the nuclear localization of p24. A long disruption of the ER will have many pleiotropic effects and may impede p24 transport indirectly.

Transit of p24 from chloroplasts to the nucleus?

Another question regarding p24 dual localization is whether nuclear p24 comes from the plastids or from the cytosol. Preliminary results using FRAP on transgenic p24-GFP tobacco roots suggest that p24 can transit from the plastids to the nucleus directly (Figure 4.7). In this experiment, GFP fluorescence in the nucleus was bleached, while GFP in the surrounding plastids was mostly unaffected. Following bleaching, fluorescence recovered in the nucleus but decreased in all surrounding plastids, indicating the p24-GFP transited from the plastids to the nucleus. This was only achieved once however, despite several trials, and more replicates need to be performed. The difficulty lies in having a fluorescent nucleus and plastids in close proximity and in the same plane. Also, all observed organelles must remain in the same plane for the duration of the experiment. Furthermore, fluorescence recovery in the nucleus could still result from p24-GFP synthesized in the cytosol. To eliminate this possibility, it would be interesting to repeat this experiment in the presence of the protein synthesis inhibitor cycloheximide. Conclusive results could also be obtained with the use of p24 fusions with photo-convertible fluorescent proteins such as the Kaede-GFP [63] and the newly described monomeric PS-CFP [64]. These proteins display an irreversible change in excitation and emission spectra following excitation with UV or violet light. Therefore, if photo-conversion is targeted to plastids containing the p24 fusion protein, then appearance of fluorescence of the shifted wavelength in the nucleus would confirm that p24 has moved from the plastids to the nucleus.

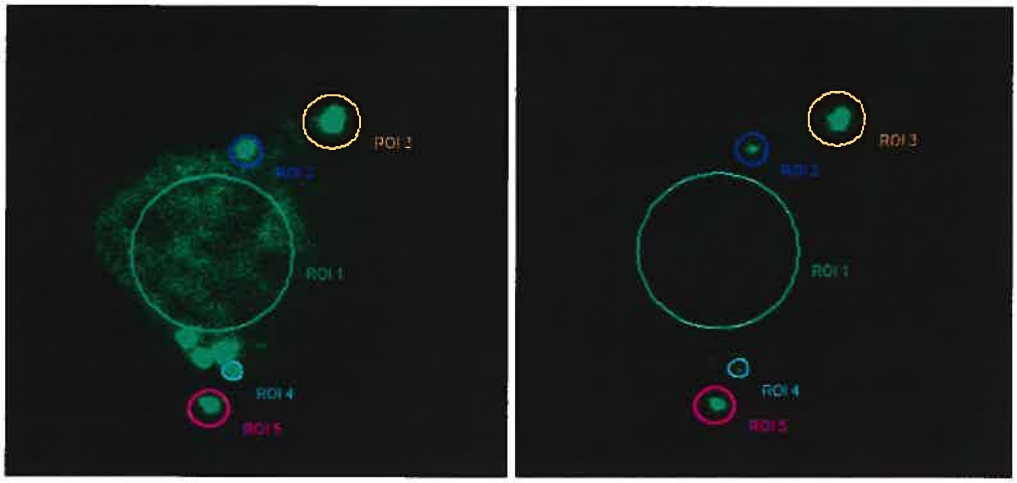
Conclusion

This chapter has presented the occurrence of p24-GFP in chloroplast stromules. These stromules are closely associated with the ER. Some plastids of cells in culture are even surrounded by ER strands, which is reminiscent of chloroplast ER found in some algae. While p24 is mostly found

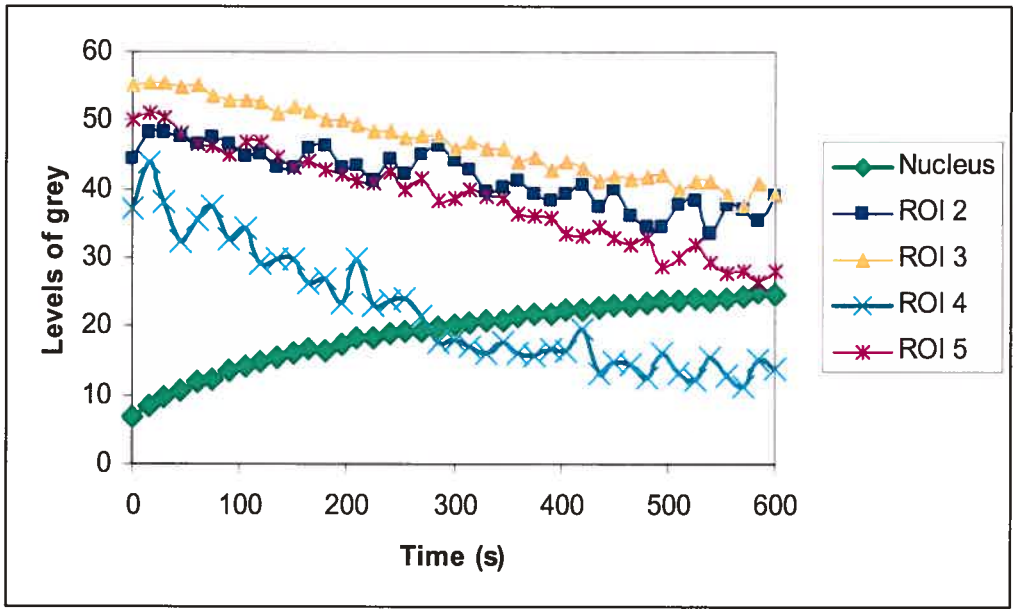
Figure 4.7

Fluorescence recovery after photobleaching. LSCM of a transgenic p24-GFP tobacco root cell. The top left panel represents the cell before bleaching. The right panel represents the cell after bleaching, but before recovery. Regions of interest (ROI) are circled and numbered 1-6. ROI 1 corresponds to the nucleus, while ROIs 2-5 are surrounding plastids. GFP fluorescence is pseudo-coloured green. The bottom graph shows the change in fluorescence intensity for each of the ROIs.

Note: the bleached area was square and slightly larger then ROI 1.



Pre-Bleach **Post-Bleach**



in plastids, nuclear localization could be induced by cytokinin treatment of cells in culture. The mechanism of p24 nuclear localization is not clear, but preliminary FRAP results suggest that p24 is transported from surrounding chloroplasts.

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Chapter V

Discussion

As sessile organisms, plants must cope with sometimes adverse environmental conditions. They have therefore evolved very sensitive mechanisms to perceive changes in their environment. Responses to such changes depend on the metabolic and developmental state of the plant and are finely tuned to balance protection with other energy demanding activities such as growth and reproduction.

Further metabolic complexity in plants results from the coexistence of heterotrophic and autotrophic tissues in the same organism. Carbon fixed in photosynthetic "source" tissues needs to be transported to heterotrophic "sink" tissues to ensure balanced growth. This process is tightly coordinated and therefore requires extensive signalling throughout the plant. Signalling is also required within cells, to ensure equilibrium between carbon fixation, usage and storage.

This thesis began with the identification of a protein (p24) that binds the promoter element of the pathogenesis related gene *PR-10a* in potato tubers. Major findings presented here include the fact that *p24* is an evolutionarily conserved gene that encodes a protein with a chloroplast transit peptide. This protein is localized in plastids and nuclei and appears to regulate stress-dependent gene expression in both compartments, contributing to signalling between them. The mechanism for dual localization has not been elucidated but appears to depend on plant hormones. Furthermore, p24 is present in chloroplast stromules that associate with ER membranes.

Role of PR-10

PR-10 proteins have no known function though it has been suggested that they are ribonucleases [1, 2]. This conclusion is disputed however. Two other studies have shown that PR-10 is a cytokinin-binding protein [3, 4]. It is not clear whether PR-10 would be a cytokinin transporter or if it would be sequestering these hormone molecules and therefore inhibiting their signalling. Finally, PR-10 has been crystallized bound to deoxycholate [5]. This molecule is not found in plants, but it is highly similar to the plant

hormones brassinosteroids and it was suggested that the latter are the physiological ligands of PR-10 [5]. Brassinosteroids and PR-10 are both present in high concentrations in pollen. It was hypothesized that PR-10 proteins are steroid carriers and allow the presentation of these hydrophobic hormones to their receptors [5]. PR-10 proteins might bind both cytokinins and brassinosteroids because they appear to possess two distinct ligand-binding sites [4].

Cytokinins in induction of sink activity

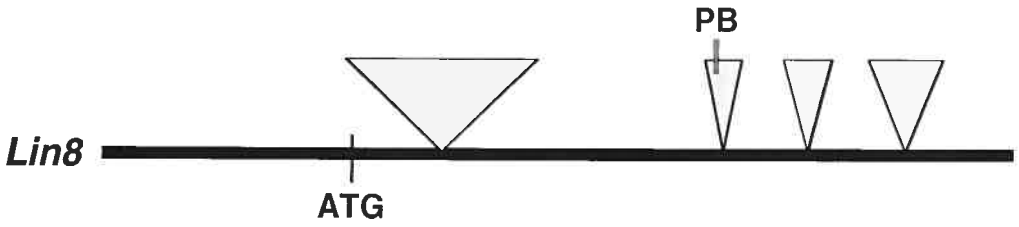
Cytokinins are known to induce sink activity in plant tissues and to delay senescence, through the induction of genes coding for extracellular invertases and sugar transporters [6, 7]. Extracellular invertase genes are also induced by pathogens [8]. These enzymes are responsible for conversion of sucrose to glucose and fructose. As such they are responsible for the local assimilation of sugars transported throughout the plant [8]. Interestingly, a putative p24 binding site can be found in the promoter (position -373) of the *Lin6* extracellular invertase gene from tomato (Figure 5.1). In addition, 5 PB sites are found in the first intron of *Lin6*, and one of these is 12 nucleotides from the left border of the intron. It is therefore possible that p24 contributes to the transcriptional activation and/or post-transcriptional processing of *Lin6*. In contrast, no PB sites are present in the promoter of the upstream *Lin8* gene, and a single PB site can be found in the second intron of that gene. *Lin6*, but not *Lin8*, is induced by brassinosteroids [9], cytokinins, glucose, wounding and pathogen elicitors [10].

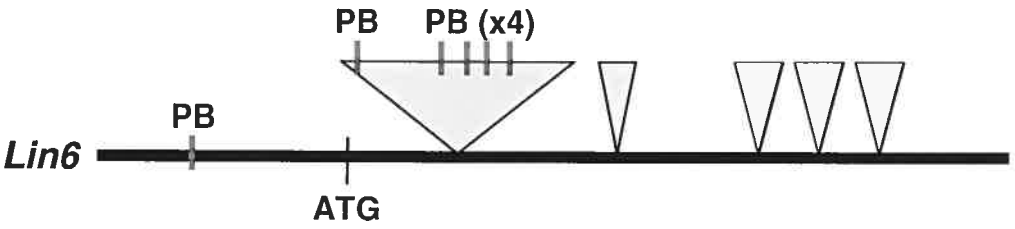
As noted in the previous chapter, cytokinins induce the nuclear localization of p24 in plant cell cultures. This allows for the intriguing possibility that p24 participates in the induction of sink activity by cytokinins, possibly through the activation of a potato *Lin6* homologue. In support of this hypothesis, p24 antisense plants produce much smaller potato tubers than WT plants (data not shown). Expression of extracellular and vacuolar invertase, but not cytoplasmic invertase, also leads to expression of PR genes in tobacco [11]. It was suggested that sensing of sugars in the secretory pathway, if their concentration exceeds a certain threshold, leads to activation of the defence response [11].

p24 in the nucleus activates *PR-10a* in response to wounding, infection or pathogen elicitors. PR-10a might in turn promote brassinosteroid signalling.

Figure 5.1

Putative PB sites in cell wall invertase (CWInv) genes from tomato. The *Lin8* and *Lin6* genes are adjacent to each other in the tomato genome (Accession AF506004). Introns are indicated by blue triangles. The putative PB sites are indicated with red lines.


PB
Lin8
ATG


PB
PB (x4)
PB
Lin6
ATG

To that effect, it is interesting to note that cytokinin and sugar signalling appear to be upstream of brassinosteroid synthesis and signalling [12].

It is also noteworthy that *PR-10a* is mainly induced in the vasculature, where it could play a role in source-sink relations. It has been shown that modifications in the source/sink balance are associated with induction of the defence response and the establishment of SAR [11].

Model of p24 activity

Chapter III showed that p24 controls photosynthesis in a stress-dependent manner. One possibility is that this serves to maintain the production of sugars and energy in source tissues to support defence activities in infected tissues that are converted to sinks. Figure 5.2 illustrates the possible roles and localization of p24 throughout the plant. Under stress conditions, such as infection, autotrophic cells not directly infected would have p24 in the chloroplasts, where it would serve to maintain photosynthesis and avoid overproduction of ROS, thus protecting these cells. Conversely, in sink cells or in infected source cells converted to sinks, p24 would move to the nucleus. There, it would activate defence genes such as *PR-10a* only if a stress stimulus such as wounding or an elicitor was present. Nuclear p24 might also induce extracellular invertase to amplify the stress response.

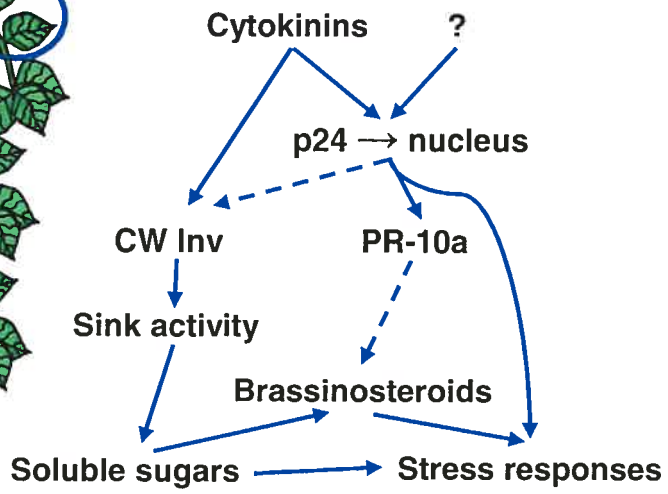
This would explain why, in potato, *PR-10a* is only strongly induced in tubers, but not in leaves, following elicitor treatment [13]. While the elicitor might be sufficient to induce the defence response, it is not sufficient to induce p24 nuclear localization. Infection with *P. infestans* does induce *PR-10a* in leaves however [13]. This makes sense because pathogens have been shown to induce sink activity [8, 14]. This source-sink transition would presumably cause p24 nuclear localization and elicitors from the pathogen would then promote induction of *PR-10a*. While p24 was observed in leaf cell nuclei using biochemical methods (see Figure 3.2), it should be emphasized that we cannot determine the cell type or the abundance of p24 in these nuclei. The amount of tissue required to detect p24 suggests that it is of very low abundance in leaf cell nuclei.

Cytokinins and source-to-sink transitions are not likely to be the sole determinants of p24 nuclear localization however. Indeed, in the xylogenesis assay presented in chapter IV, only a fraction of the cells in culture exposed to cytokinins and high sucrose concentrations showed p24-GFP nuclear

Figure 5.2

Proposed model of p24 action and localization. p24 is always present in plastids where it has a protective function by regulating photosynthesis. Nuclear localization of p24 depends on a certain developmental program requiring cytokinins and another unknown signal. This developmental program probably coincides with the activation of sink activity. Upon nuclear localization, a stress signal causes p24 to induce defence gene expression. PR-10a is thought to be a brassinosteroid carrier, and both brassinosteroids and soluble sugars are known to participate in defence signaling. Solid arrows: demonstrated links. Dotted arrows: proposed links.

Source tissues	
Non-infected	Infected
p24 in the chloroplast Maintenance of photosynthesis	p24 in the nucleus Increased sink activity Increased defence activities



Sink tissues	
Non-infected	Infected
p24 in the plastids or in the nucleus Maintenance of ATP production or induction of sink activity	p24 in the nucleus Increased sink activity Increased defence activities

localization. Also, most root cells express the genes for cytokinin receptors [15], but only a few cells from transgenic roots showed p24-GFP in the nucleus. Another signal, probably developmental, is therefore required for translocation of p24 to the nucleus.

Similarities between p24 and animal pro-apoptotic proteins

In plastids, our results suggest that p24 could control cyclic electron flow by affecting the activity of the plastid NDH enzyme (see Figure 3.18). NDH is homologous to the mitochondrial respiratory chain complex I [16, 17]. Strikingly, animal AIF, a protein shown to have dual mitochondrial/nuclear localization, was shown to be important for the activity of mitochondrial complex I under normal conditions [18]. It is thought that AIF protects the cell, through its intrinsic NADH oxidase activity, by maintaining complex I activity and providing resistance to oxidative stress [18]. This is in direct contrast to the apoptotic activity of AIF in the nucleus.

It is interesting that complex I and its plastid homologue (ie. NDH) could both be targeted by proteins that also localize to the nucleus, though the significance of this is not clear. AIF, once translocated to the nucleus, induces apoptosis [19]. There is no evidence yet that nuclear p24 contributes to programmed cell death. Like p24, AIF associates with nuclear DNA, though it is in association with endonuclease G, and AIF does not appear to be a transcriptional regulator [20]. In conclusion, although there are strong similarities between these two systems, there are also significant differences.

In that sense, p24 is more similar to the transcriptional regulator p53. p53 is also a tetramer [21]. Both proteins are present in two locations in the same cells [22 and Chapter III]. Interestingly, a fraction of p53 is at the surface of mitochondria while a certain number of p53 molecules are inside mitochondria [22]. In some confocal pictures of plant cells expressing p24-GFP, it sometimes appears that some of the p24-GFP speckles are on the outside of chloroplasts, rather than inside. It would be interesting to purify these intact chloroplasts and treat them with proteases to see if a fraction of p24-GFP disappears, which would mean that it was at the surface of the chloroplast.

In contrast to p24 however, there is no evidence that p53 affects organelle gene expression, though a recent study has identified a putative p53-binding site in the human mitochondrial genome [23]. This site is

recognized by p53 when inserted into the nucleus [23]. It has also been found that p53 interacts directly with mitochondrial manganese superoxide dismutase and inhibits its activity [24]. The possibility that p24 has other non-transcriptional effects in chloroplasts also cannot be excluded at this stage.

p24: prokaryotic or eukaryotic transcription factor?

The DNA-binding and transcriptional activities of p24 in both plastids and the nucleus poses another challenging question. How can p24 be a transcriptional activator in both a prokaryotic and an eukaryotic context? As mentionned in chapter III, there are at least two types of polymerases operating in plastids and p24 appears to contribute to NEP-dependent transcription. The PEP polymerase is of prokaryotic origin, while the NEP polymerase has the same characteristics and is sensitive to the same inhibitors as the viral T7 polymerase [25]. A study in animal cells showed that a reporter gene under the T7 promoter, inserted into the nuclear genome, was transcribed just as efficiently in the presence or absence of a T7 polymerase transgene [26]. This shows that T7 promoters are recognized by the eukaryotic nuclear transcription machinery. In that context, it is possible that different proteins in each compartment serve as bridges between the DNA binding proteins (such as p24) and the respective polymerases.

It is interesting that other dually-localized plant proteins, such as SEBF and SigA2 are also involved in transcription. p24 likely acts in concert with these proteins to control gene expression in chloroplasts and nuclei, and the communication between these two compartments.

Future prospects

The study of p24 has proven both challenging and rewarding. Like AIF and p53, p24 appears to have many functions and cellular locations. A model is emerging in which p24 has a protective function in the chloroplast, by maintaining photosynthesis, and a role in pathogen defence in the nucleus. These two roles are probably complementary, especially when considered in the context of the whole plant. Finally, it is likely that p24 has several other as yet unidentified functions.

A number of questions warrant further study in the near future. First, it is still unclear how p24 moves to the nucleus. As mentionned above, this could be addressed by using fusions of p24 with photo-convertible fluorescent

proteins. It might also be informative to try different p24 mutations in the xylogenesis assay to see which domains of p24 are involved in nuclear localization. Inhibitors of the ER or golgi apparatus, such as Bfa or NDGA, could test whether p24 nuclear localization depends on the secretory pathway. FRAP also holds promise to test whether p24 moves from the chloroplasts to the nucleus. Use of cycloheximide would ensure that p24 accumulating in the nucleus does not result from *de novo* synthesis in the cytosol.

Another important issue is the role of dual localization. The double role of p24 is most likely to ensure a coordinated response to stress. Nonetheless, the identification of mutations in p24 that lead to single localization would give clues as to the precise role of p24 in each compartment. Furthermore, such mutations would allow the study of chloroplast-nucleus signalling.

The role of p24 in source-sink relations is another attractive avenue of research. Given the putative PB sites in a stress-responsive extracellular invertase gene (Figure 5.1), it would be interesting to see if p24 affects the expression of this gene and if this depends on cytokinins. Levels of soluble sugars and starch in p24 transgenic plants could also be determined. In addition, to confirm that cytokinins are responsible for changes in p24 localization, cytokinin-insensitive mutant plants or plants with defects in cytokinin synthesis could be tested for p24 nuclear localization.

Finally, the subject of how p24 controls gene expression in two compartments could have an important impact on evolutionary theories. Is the *PR-10a* promoter recognized by a prokaryotic transcription system? Do chloroplast NEP promoters function in the nucleus? Did the nucleus inherit promoter sequences and coactivator genes from the cyanobacterial endosymbiont?

Concluding remarks

Plant responses to their environment are finely tuned to both internal and external stimuli. This coordination depends on extensive signalling throughout the plant and within each cell. The discovery of proteins that have multiple roles and cellular locations has offered a potential mechanism by which different compartments could respond to the same stimuli in a coordinated fashion. The novel transcriptional activator p24 appears to

synchronize photosynthetic activity with the energetic demands of biotic and abiotic stress defence.

This thesis offers a framework by wich intracellular signalling and stress defence can be studied in the context of whole-plant biology. Doing so will provide one with, as Barbara McClintock would have said, a better "feeling for the organism".

Note added in proof

A recent publication reported the purification of two members of the p24 family as part of a protein complex corresponding to a membrane-bound, transcriptionally active chromosome (TAC) in plastids of both Arabidopsis and mustard [27]. Other proteins in this complex included subunits of PEP, as well as a DNA gyrase, elongation factor Tu, several ribosomal proteins, superoxide dismutase (SOD), phosphofructokinase, thioredoxin and others. This mix of proteins suggests that plastid transcription might be linked to translation and be controlled by ROS, sugars and redox signals. It will be interesting to determine the role of p24 in these processes.

Finally, the intracellular localization of all three Arabidopsis members of the p24 family has been elucidated [28]. Both At1g14410 and At2g02740 were found to be targeted to the chloroplasts when fused to GFP, while At1g71260 was found in mitochondria. The work presented in this thesis does not include the study of the potato homologue of At1g71260, and it would be interesting to see if this protein also participates in stress-dependent gene expression in mitochondria.

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Annex 1

A perl computer program to search for potential transcription factor binding sites in a given sequence

```
# 1 !/usr/bin/perl -w
print "This is a program to look for DNA motifs in a
sequence\n\n";

use strict;
use warnings;
my $DNAfilename = '';
my @DNA =();
my $p24_search_results='p24results.txt';

# 10 Ask for file name
print "Please type the filename of the DNA sequence data:";
$DNAfilename = <STDIN>;

# 14 Remove the new line from the DNA filename;
chomp $DNAfilename;

# 17 Open the file or exit
unless ( open(DNAFile, $DNAfilename) ) {
     print "Cannot open file \"$DNAfilename\"\n\n";
     exit;
}

# 23 Read the file and store the data into array @DNA
@DNA = <DNAFile>;

# 26 Close the file
close DNAFile;

    # 29 Declare and initialize variables
    my $sequence = '';

    foreach my $line (@DNA) {

        # 34 discard blank line
        if ($line =~ /^\s*$/) {
            next;

        # 38 discard comment line
        } elsif($line =~ /^\s*#/) {
            next;

        # 42 discard fasta header line
        } elsif($line =~ /^>/) {
            next;

        # 46 keep line, add to sequence string
        } else {
            $sequence .= $line;
        }
```

```
    }

    # 52 remove non-sequence data (in this case, whitespace)
from $sequence string
    $sequence =~ s/\s//g;
    # remove digits
    $sequence =~ s/[0-9]//g;

# 57 Check for p24 motif
my $motif1 = '[at]gtc[at][at][at][at][at]';

#60 Look for motif
if ($sequence =~ /$motif1/) {
 print "I found it!\n\n";
} else {
    print "I couldn\'t find it.\n\n";
}

sub match_positions {
my ($motif, $dnaseq) = @_;
     my @positions = ();
     while ($dnaseq =~ /$motif/ig) {
     push (@positions, pos($dnaseq) - length($&));
     }
     return @positions;
}
open (OUTFILE, ">>$p24_search_results");
my @locations=();
my $array='';
my $x='';
my $n='';
@locations = match_positions($motif1, $sequence);
if (@locations) {
     print "The motif $motif1 was found at positions: \n";
     print join(" ", @locations), "\n";
# 83
$n=0;
foreach $x(@locations) {
if (($x-150)<0) {
     print "Position is too close to beginning";
     $x=150;
} else {
     $x=$x;
     }
     $array = substr($sequence, $x-150, 300);

     print (OUTFILE '> ');
     for ($n > length(@locations)) {
     print (OUTFILE $locations[$n]);
     }
     print (OUTFILE " is:\n");
     for (my $pos = 0 ; $pos < length($array); $pos += 80)
{
          print (OUTFILE substr($array, $pos, 80), "\n");
     }
     print (OUTFILE "\n");
```

```
      ++$n;
}
} else {
      print "The motif $motif1 is not in this DNA\n";
}

# 57 Check for p24 motif
my $motif2 = '[at][at][at][at][at]gac[at]';

#60 Look for motif
if ($sequence =~ /$motif2/) {
 print "I found it!\n\n";
} else {
    print "I couldn\'t find it.\n\n";
}

my @locations2=();
my $array2='';
my $y='';
my $m='';
@locations2 = match_positions($motif2, $sequence);
if (@locations2) {
      print "The motif $motif2 was found at positions: \n";
      print join(" ", @locations2), "\n";
# 83
$m=0;
foreach $y(@locations2) {
if (($y-150)<0) {
      print "Position is too close to beginning";
      $y=150;
} else {
      $y=$y;
      }
      $array2 = substr($sequence, $y-150, 300);

      print (OUTFILE '> ');
      for ($m > length(@locations2)) {
      print (OUTFILE $locations2[$m]);
      }
      print (OUTFILE " is:\n");
      for (my $pos2 = 0 ; $pos2 < length($array2); $pos2 +=
80) {
            print (OUTFILE substr($array2, $pos2, 80), "\n");
      }
      print (OUTFILE "\n");

      ++$m;
}
} else {
      print "The motif $motif2 is not in this DNA\n";
}
#104
      close OUTFILE;
exit;
```