Studies on expression of senescence-associated genes and physiological function of protein products in tobacco plants

Seung-Hwan Yang

2002

Studies on expression of senescence-associated genes and physiological function of protein products in tobacco plants

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Seung-Hwan Yang
The Research and Education Center for Genetic Information
Nara Institute of Science and Technology
(Professor Hiroshi Sano)
January, 2002

Abstract

Senescence is a highly organized process under genetic control that is not only age-dependent, but is also induced by environmental conditions such as shading, low temperature, dehydration and pathogen infection. During senescence, chloroplasts and other cellular organelles are dismantled, and macromolecules such as chlorophyll, lipids, proteins and nucleic acids are degraded. Many senescence-associated genes (SAGs), which are defined as up-regulated ones during senescence, have been isolated and characterized. However, many SAGs have functions that remain unknown.

In this study, I describe analysis of expression and physiological roles of SAGs from tobacco plants. This thesis consists of 4 chapters; Chapter I describes isolation and characterization of *Ntdin* encoding a protein involved in molybdenum metabolism; Chapter II describes isolation and characterization of *tbzF* and *tbz17*, genes encoding bZIP transcriptional activators; Chapter III describes promoter analysis of *tbzF*; Chapter IV describes isolation of TBZF interaction factors by yeast two-hybrid system.

In Chapter I, I characterized *Ntdin*, of which transcripts are induced by darkness and during senescence. *Ntdin* is related to Radish *din1* and Arabidopsis *sen1*. Its product, Ntdin, consists of 185 amino acids, and shows 56.8% and 54.2% similarities to Atsen1 and Rsdin1, respectively. It also shows some similarity with the C-terminal region of *N. plumbaginifolia* Cnx5, which has a function in molybdenum cofactor (Moco) biosynthesis. Ntdin-GFP fusion protein was found to localized in chloroplasts. Transcripts of *Ntdin* are induced by sulfate and nitrate but not by phosphate. Tobacco plants expressing an antisense *Ntdin* gene showed decreased molybdoenzyme activity due to a deficiency of Moco, suggesting that Ntdin functions in Moco biosynthesis.

In Chapter II, I characterized two bZIPs, TBZF and TBZ17, belonging to the LIP19 subfamily. Transcripts of *tbzF* were detected at high level in senescing leaves and flowers. In contrast, *tbz17* transcripts accumulated in aging leaves but not in flowers. *In situ* hybridization

analysis revealed transcripts of *tbzF* and *tbz17* to be predominantly located in guard cells and vascular tissues of senescing leaves. TBZF and TBZ17 show 73% identity each other and are located in the nucleus. Bacterially expressed proteins preferentially bound to DNA fragments spanning A-box/G-box and C-box/G-box hybrid motifs, and showed transactivation activity in co-transformed tobacco BY2 cells, confirming that they function as transcriptional activators. These results suggest that TBZF and TBZ17 are both involved in controlling transcription of genes functioning in guard cells of senescing leaves and that TBZF bifunctionally acts in floral development.

In Chapter III, I examined whether or not expression of *tbzF* is differentially regulated in response to different physiological conditions. I isolated a 1740 bp promoter region, and fused it to the β-glucuronidase (GUS) reporter gene, which was introduced into tobacco plants. Resulting transgenic plants exhibited GUS activity in the stomatal guard cells of senescing leaves and in flower buds, and also in ABA- and ethylene-treated young leaves. To identify *cis*-responsive regions, four deletion constructs were made and transformed into tobacco plants. GUS assays revealed that the *cis*-element for senescence is located on a different region from that for flower development.

In Chapter IV, I describe identification of genes encoding proteins that interact with TBZF by yeast two-hybrid screening. Among several clones, one particular clone was found to encode a protein that predominantly interacts with TBZF, and designated NtTIP1 (*N.tabacum* TBZF interacting protein). *NtTIP1* encodes a 12.5-kD basic protein, which is localized in the nucleus, and is expressed upon water deficit and during fruit ripening. Expression of *NtTIP1* homologous was also seen in different tissues and in response to cold, ABA and various other stress treatments.

Based on these observations, I concluded that Ntdin functions in providing molybdenum ions to senescence-associated proteins such as NR, and that, TBZF, TBZ17 and NtTIP1 are key regulators of multiple genes, of which activation is important for senescence processes.

Contents

Abbreviations

a.a.	amino acids
ABA	abscisic acid
ATA	aurin tricarboxylic acid
ATP	adenosine 5'-triphosphate
BA	N 6-benzylaminopurine
bp	base pairs
BY2	Bright Yellow 2
CaMV35S	cauliflower mosaic virus 35S
cDNA	complementary DNA
CTAB	hexadecyltrimethylammonium bromide
d	day
Da	dalton
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	1,4-dithio-DL-threitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
EtOH	ethyl alcohol
GFP	green fluorescent protein
GST	glutathione S-transferase
GUS	β-glucuronidase
h	hour
IAA	indole-3-acetic acid

JA	jasmonic acid
kb	kilo base
kD	kilo dalton
LB	Luria broth
LUC	luciferase
MeJA	methyl jasmonate
min	minute
mRNA	messenger RNA
MS	Murashige and Skoog
nt	nucleotides
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	PCR in conjunction with reverse transcription
SSC	sodium chloride / trisodium citrate
TAIL-PCR	thermal asymmetric interlaced PCR
TBE	Tris-borate, EDTA buffer
TE	tris / EDTA
Tris	tris(hydroxymethyl)aminomethane
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-bufferd saline
PCR	polymease chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate buffer
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)methylglycine
UTR	untranslated region
UV	ultraviolet

General introduction

What is senescence?

Senescence in plants is the sequence of biochemical and physiological events comprising the terminal phase of biological development, from the mature, fully expanded state until death of single cells, organs or even whole plants during their life cycle (Smart, 1994). It presages death and occurs at various levels of biological organization including the whole plant, as in monocarpic senescence, organs as in foliar, floral and fruit senescence, tissues as in the differentiation of tracheary elements during genesis of the vascular system, and individual cells. In fact, cellular senescence is very common in the plant kingdom and appears to be an integral feature of plant growth and development. The abscission of flowers, leaves and fruits, for example, is attributable to senescence of a specialized layer of cells in the abscission zone (Noodén, 1988; Gan and Amasino, 1997; Noh and Amasino, 1999).

Generally, apoptosis refers to programmed death of small numbers of animal cell, and it shows some special features at the cell level. Some senescing plant cells show some symptoms typical of apoptosis, while others do not (Noodén et al., 1997). There are three distinct forms of cell death - apoptosis, atrophy and differentiation (Bowen, 1993). Plant senescence appears to resemble most closely differentiation to death in animals.

Senescence can be particularly dramatic in monocarpic plants, those that reproduce once and then die at the end of their reproductive phase (Noodén and Leopold, 1978). Usually, monocarpic plants stop replacing their old vegetative organs, for example, leaves, during reproductive development, and the remaining organs are signalled to die (Noodén, 1980). In monocarpic plants, the reproductive structures often govern senescence of the whole plant with especially striking effects on the leaves.

Senescence may be thought of as the 'last will and testament' of a leaf, in which bequests' of nutrients are made to the rest of the plant. As a leaf passes the peak of assimilatory capacity, mesophyll tissue begins to loss its greenness and turn yellow or red (Smart, 1994). In forests of deciduous trees, the autumn colors that develop during leaf senescence are of

great aesthetic value. The colour change is due both to preferential degradation of chlorophylls compared with carotenoid, and the synthesis of new compounds such as anthocyanins and phenolics (Matile, 1992). Furthermore, this process is also of great practical value because during leaf senescence, nutrients are recycled to other parts of the plant. For example, nitrogen from leaves of deciduous trees is used for the synthesis of storage proteins in stems that will support growth during the following spring (Clausen and Apel, 1991; Gan and Amasino, 1997). However, in an agricultural setting, leaf senescence may limit yield in certain crops. Senescence also contributes to the post harvest loss of vegetable crops. Therefore, studying leaf senescence will not only contributes to our knowledge about this fundamental developmental process, but may also lead to ways of manipulating senescence for agricultural applications.

Regulation of senescence

Leaf cells undergo the sequential disorganization of cellular organelles and dramatic changes in cellular metabolism during leaf senescence (Noodén, 1988; Smart, 1994; Feller and Fisher, 1994; Matile et al., 1996). Metabolic changes include loss of photosynthetic activities and hydrolysis of macromolecules that accumulate during the growth phase (Fig. 1). Degenerative activities are often concomitant with the massive remobilization of the hydrolyzed compounds to the growing parts of plants such as young leaves and developing seeds. Thus, leaf senescence, although deteriorative in nature, is a critical process for the fitness of plants and is regarded as an evolutionarily acquired genetic process (Gay and Thomas, 1995; Nam, 1997).

Leaf senescence is the sequence of degradative processes leading to the remobilization of nutrients and eventual leaf death. The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation, and chlorophyll degradation (Smart, 1994). Total RNA levels decline during senescence as RNase activity increases (Blank and McKeon, 1991). Chloroplasts are one of the earliest sites of catabolism, while mitochondria remain intact until late in the senescence process in order for respiration to

continue (Smart, 1994). Plant hormones are involved in regulating the senescence process, with cytokinins delaying senescence, ethylene modulating the timing of senescence, and the other hormones playing less prominent roles (Smart, 1994). Leaf senescence, like other developmental processes, is actively regulated by differential gene expression. Transcript levels for photosynthetic genes such as rbcS (small subunit of Rubisco) and cab (chlorophyll a/b-binding protein) decline (Bate et al., 1991), while other genes become activated (Buchanan-Wollaston, 1997; Weaver et al., 1997).

Although senescence occurs in an age-dependent manner in many species, the initiation and progression of senescence can be modulated by a variety of environmental (external) and autonomous (internal) factors (Noodén, 1988). The external factors include temperature, nutrient deficiency, shading, drought conditions, ozone, wounding, and pathogen infection. The internal factors include age, plant growth regulators, reproduction, and cellular differentiation (Thomas and Stoddart, 1980; Hensel et al., 1993).

Among the external factors, limited water and nutrient availability are major factors that adversely affect plant life in many ecosystems. Plants have evolved mechanisms by which leaf senescence can be induced by these stresses to reallocate nutrients to reproductive organs and to eliminate water consumption by older, less productive leaves. This regulation of leaf senescence has an obvious adaptive value, allowing the plant to complete its life cycle even under stressful conditions (Gan and Amasino, 1997).

Among these internal factors, plant hormones have been characterized most thoroughly at the molecular and physiological levels. For example, abscisic acid (ABA) regulates many aspects of plant growth and development, including tolerance to adverse environmental conditions as well as seed maturation and dormancy (Leung et al., 1997). ABA also induces leaf abscission, flower senescence, and leaf senescence (Zeevaart and Creelman, 1988). Ethylene has been known as an endogenous modulator of senescence, including fruit ripening and flower and leaf senescence (Abeles et al., 1988). Indeed, several ethylene-insensitive mutants of *Arabidopsis*, *etr1*, *ein2*, and *ein3*, were shown to have delayed leaf senescence symptoms (Zacarias and Reid, 1990; Grbi and Bleecker, 1995; Chao et al., 1997; Oh et al., 1997). Jasmonates induce plant defense responses and mechanical wounding (Bell et al.,

1995; Reymond and Farmer, 1998). In addition, jasmonates are important cellular regulators of diverse developmental processes, such as flower and fruit development, leaf abscission, and senescence (Creelman et al., 1992). Other previous analyses also showed the effectiveness of ABA, ethylene, and methyl jasmonate (MeJA) in promoting leaf senescence in *Arabidopsis* (Oh et al., 1996, 1997; Weaver et al., 1998).

Central pathway of senescence

Senescence is a highly regulated process, during which new metabolic pathway are activated and others are turned off. A scheme outlining the likely steps in the pathway leading to leaf senescence is shown in Figure 2. Senescence can be initiated by a wide variety of factors (Noodén, 1988; Gan and Amasino, 1997), and these may feed into the same pathway.

In this scheme, signal transduction cascades linked to various triggers such as hormonal and environmantal stimuli lead to an initiation of many genes, followed by the transition to a phase of controlled redifferntiation of cell structures and remobilizing of materials. Finally, a termination phase ensues, in which many of the features of generalized plant cell death (PCD) can be recognized. The integrity of subcellular membranes and the compartmentation of biochemical pathway are preserved until late in the terminal phase (Noodén et al., 1997; Buchanan-Wollaston and Ainsworth, 1997).

Many of the enzymes that increase during senescence are clearly secondary or peripheral to senescence. These include the enzymes required to metabolize the amino acids and lipids released and to convert them to transfortable forms. In addition, some proteins such as metallothionein and peroxidase may be needed to protect the normal cell functions against the metal ions and peroxides released (Buchanan et al., 1997, Nam, 1997). In addition, one can ask if there is one senescence process or several different processes occurring in different tissues or circumstances, e.g., 'normal' vs stress-induced senescence (Noodén, 1988). Although breakdown of the chloroplast (CP) is an important aspect of the senescence syndrome, the loss of the CPs themselves may not kill the leaf cells (Noodén, 1988).

Gene expression in senescence

Leaf senescence is accompanied, and perhaps driven, by changes in gene expression; the majority of genes that are active in non-senescing leaves such as those involved in photosynthesis are down-regulated while a subset of genes are up-regulated during senescence. Studies involving inhibitors of RNA and protein biosynthesis have shown that activation of new genes is required for leaf cells to undergo senescence (Noodén, 1988; Gan and Amasino, 1997). Therefore, efforts have been made to isolate genes whose transcript abundance increases during leaf senescence, but only a limited number of SAGs (senescence-associated genes; defined as genes with expression up-regulated during leaf senescence) have been identified from a number of plant species, such as *Arabidopsis thaliana* (Hensel et al., 1993; Taylor et al., 1993; Lohman et al., 1994; Oh et al., 1996; Park et al., 1998), *Brassica napus* (Buchanan-Wollaston and Ainsworth, 1997), asparagus (King et al., 1995), barley (Kleber-Janke and Krupinska, 1997), maize (Smart et al., 1995), radish (Azumi and Watanabe, 1991), and tomato (Drake et al., 1996; John et al., 1997).

The encoded gene products are principally involved in degradation or remobilization of biomolecules, but are also involved in protecting cell viability for completion of the senescence process. Many SAGs have functions that remain unknown. Some are possibly involved in triggering senescence or controlling the progression rate of senescence. Analysing the various modes of SAGs regulation should provide insights into the molecular mechanisms of the leaf senescence programme.

About 50 cDNA have been assigned possible functions in senescence on the basis of sequence homology, even though in only a few cases have these functions been confirmed biochemically or physiologically. Some of the gene that are up-regulated during senescence are listed in Table 1. Among the identified senescence-induced genes are genes encoding proteases, RNases, Gln synthetase, metallothioneins, protease regulators, ACC oxidase, lipases, glyoxylate cycle enzymes, catalase, endoxyloglucan transferase, pathogenesis-related proteins, ATP sulfurylase, glutathione *S*-transferase, Cyt P450, and polyubiquitin (Buchanan and Wollaston, 1997; Weaver et al., 1997; Bleecker, 1998).

SAGs may be defined as genes whose expression increases during natural leaf senescence (i.e. developmental age-dependent senescence) relative to other developmental stages of the leaf (Fig. 1). Leaf senescence can be initiated or modulated by several internal and external factors as well as in an age-dependent manner. In contrast, many plant genes that respond to environmental factors may not be associated with leaf senescence and are regarded as genes specific to the environmental factors (Nam, 1997).

Genes encoding proteolytic enzymes, such as cysteine protease-like genes, is being expanded continuously (Smart, 1995; Drake et al., 1996; Tournaire et al., 1996). The role of proteolytic degradation in leaf senescence is illustrated by the biochemical identification of a cysteine protease and a serine protease that catalyze the degradation of Rubisco (ribulose-1,5-biphosphate carboxylase/oxygenase), a major leaf protein undergoing degradation during leaf senescence (Yoshida and Minamikawa, 1996), and by the immunological identification of alkaline endopeptidases that increase during leaf senescence (Morris et al., 1996). The genes in the ubiquitin pathway such as those encoding polyubiquitin in potato (Garbarino et al., 1995) and *Arabidopsis* (Park et al., 1998), are also induced during leaf senescence.

Other SAGs involved in hydrolytic activities include those that encode RNases such as the bean ribonuclease-like pathogenesis-related (PR) protein gene, *Ypr10* (Crowell et al., 1992; Walter et al., 1996). RNases have been suggested to function in phosphate remobilization during senescence. A pumpkin gene for a key glyoxysomal protein, 3-ketoacyl-CoA thiolase, which may be involved in the remobilization of fatty acids, is induced during leaf senescence like other glyoxysomal protein genes (Graham et al., 1992). A gene for cytosolic glutamine synthetase induced during senescence may function to remobilize the nitrogen compounds released during senescence (Watanabe et al., 1994; Sakurai et al., 1996). Other SAGs include a β-glucosidase gene (Callard et al., 1996) and a gene encoding a peptide related to an endoxyloglucan transferase that may function in cell wall metabolism (Nam, 1997; Park et al., 1998).

Many stress-related genes are also induced during senescence. These include metallothionein-like genes (Buchanan and Wollaston, 1997; Hsieh et al., 1995; Thomas et al., 1997), that may be involved in the chelation of metal ions released during cellular

degradation. The genes involved in the oxidative stress response are also induced, including the genes for Fe^{2+}-ascorbate oxidase (Callard et al., 1996), anionic peroxidase (Tournaire et al., 1996), glutathione *S*-transferase (Jepson et al., 1994; Smart et al., 1995) and a blue copper-binding protein (Van-Gysel et al., 1993). Several pathogenesis-related protein genes are induced in senescing leaves (Walter et al., 1996; Hanfrey et al., 1996; John et al., 1997). The radish *dinl* gene (Azumi and Watanabe, 1991) and its *Arabidopsis* counterpart, *senl* (Oh et al., 1996), are induced by dark treatment. These stress-related genes may participate in protecting the cellular integrity required for progression and completion of senescence.

Thus, the SAGs identified so far include genes executing the senescence program such as genes involved in disintegration or remobilization of macromolecules, genes involved in protecting cell viability for completion of the senescence process; however, it is expected that SAGs may also include genes involved in the initiation or triggering of leaf senescence, and genes controlling the progression rate of senescence (some of the genes involved in disintegration or remobilization of macromolecules, including RNase, protease or ubiquitin pathway genes, may also function in this latter category).

Studying genes downregulated during leaf senescence may also provide some information on the molecular mechanism of leaf senescence (Fig. 1). Senescence may involve the downregulation of not only many metabolic enzymes but also critical components that repress the senescence program. The downregulated genes identified recently include the genes for ATP sulfurylase (Smart et al., 1995), a photosystem II 10 kDa polypeptide (John et al., 1997), and a few stromal enzymes (Crafts-Brandner et al., 1996).

Molecular mechanisms of senescence

Studying the regulation of *SAG* expression should help to decipher the molecular mechanisms of initiation and progression of leaf senescence. Current investigations of the kinetics of *SAG* expression patterns under natural and artificial senescence induction conditions suggest that the regulation of *SAG* expression is a complex process that may be controlled by an array of internal and environmental factors (Noodén, 1988; Smart, 1994; Dai

et al., 1999) through a regulatory network (Gan and Amasino, 1997). To fully understand the molecular basis of leaf senescence, it is necessary to identify genes whose products are components of the biochemical and regulatory pathways of leaf senescence.

Many *SAGs* are induced by adverse internal and external environmental factors, including the presence of heavy metals, salicylic acids, dark, senescence-affecting hormones (ethylene, abscisic acid and methyl jasmonate), wounding, heat shock and nutrient starvation (Hsieh et al., 1995; Oh et al., 1996; Chung et al., 1997; Gan and Amasino, 1997; Park et al., 1998; Weaver et al., 1997, 1998). Thus, many *SAGs* also function in the plant's response to these environmental factors. Indirectly, this may mean that the senescing cells are highly stressed and require the expression of these stress-responsive genes to cope with the stressful conditions. It is likely that many other stress-related genes are also induced during leaf senescence.

Purpose and composition of this thesis

As described above, leaf senescence is not conducted by a unique set of senescence-specific genes but primarily utilizes genes that are also involved in other cellular processes. Plant cells may have recruited these genes during the evolution of the leaf senescence program and may have modulated their expression to be senescence-associated in the leaf; however, it is certainly conceivable that leaf cells utilize genes specific to leaf senescence. It will be important to identify such genes to reveal the mechanism of leaf senescence. Such genes may include those that control the basic senescence program, that is, the age-dependent developmental program.

In this study, I describe analysis of expression and physiological roles of SAGs from tobacco plants. This thesis consists of 4 chapters; Chapter I describes isolation and characterization of *Ntdin* encoding a protein involved in molybdenum metabolism; Chapter II describes isolation and characterization of *tbzF* and *tbz17*, genes encoding bZIP transcriptional activators; Chapter III describes promoter analysis of *tbzF*; Chapter IV describes isolation of TBZF interaction factors by yeast two-hybrid system.

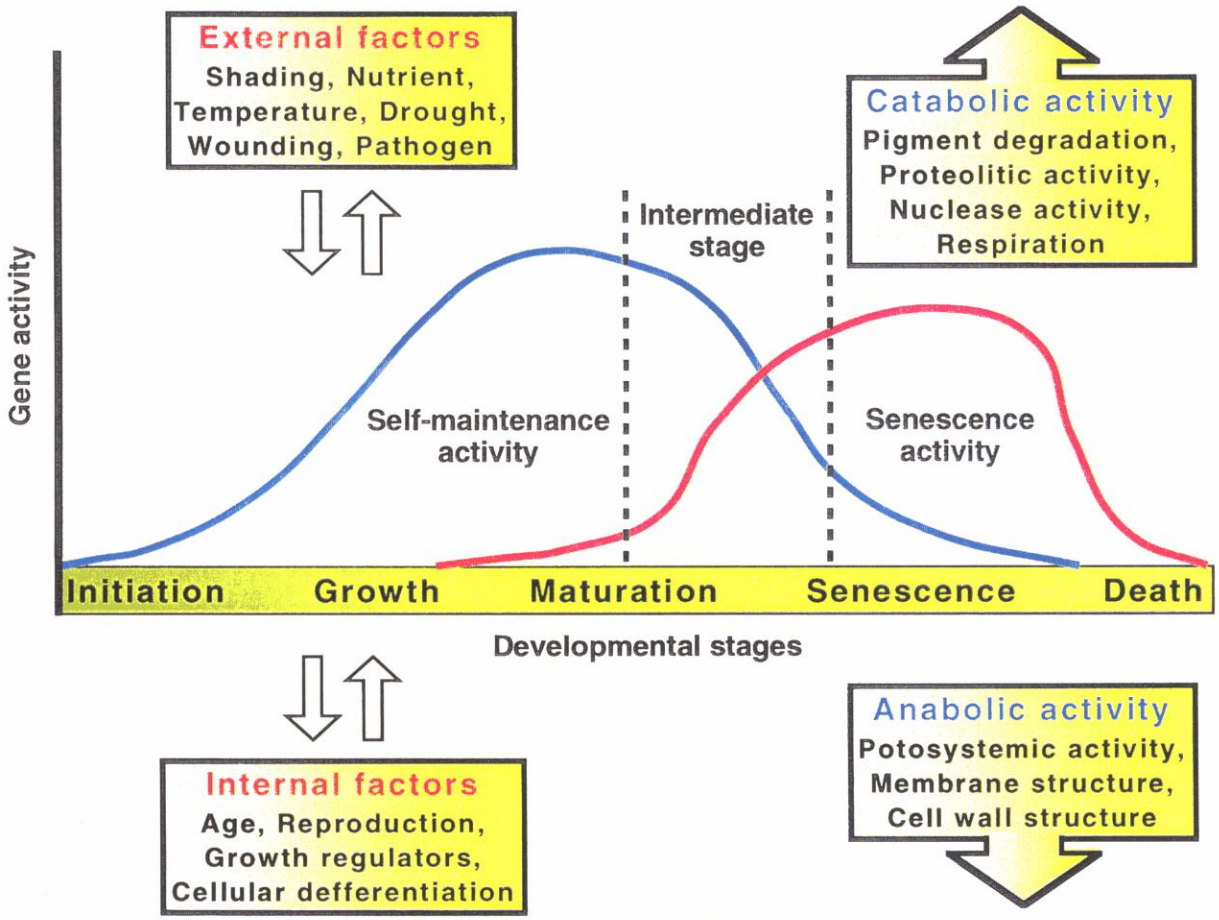


Fig. 1 A simple model for leaf growth and senescence. The regulation of senescence is viewed as a matter of balance between two antagonistic self-maintenance and senescence gene activities. The self-maintenance gene activity increases during leaf growth and declines at the senescence stage. This activity includes genes involved in many anabolic activities such as photosynthesis. Other basic cellular maintenance activities include genes associated with RNA and protein synthesis and repair activities. Gene activity associated with senescence may start to accumulate at earlier stages but the resulting symptoms are not apparent until the loss of self-maintenance gene activity at a later stage. In the intermediate stage, both activities coexist and the plant may retain self-maintenance or proceed towards senescence, depending on the conditions. Because of this, the initiation point for senescence cannot be clearly defined. The process is developmentally programmed but once a certain threshold level of senescence gene activity has been reached, the leaf cell proceeds towards death. Developmental age-dependent and environmental factors influence the timing and progression rate of senescence.

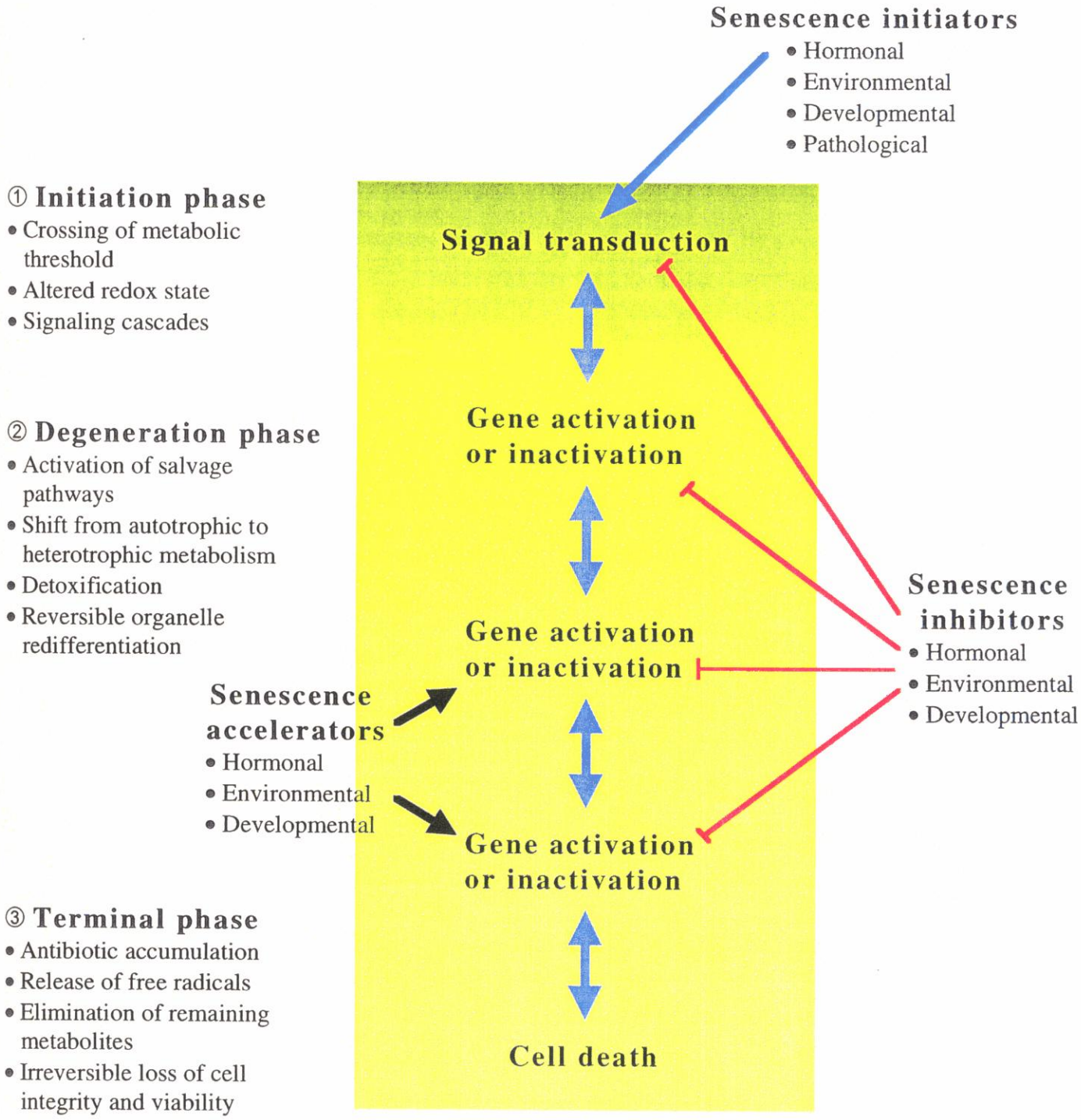


Fig. 2 A proposed scheme showing steps in the process of leaf senescence from the initiating signal to cell death.

Table 1 Cloned genes that show increased transcription during senescence.

Gene name	Possible function	Plant	Other information[1]
SAG2	Cysteine protease	*Arabidopsis*	Oryzain ϒ-like
See1	Cysteine protease	Maize	Oryzain ϒ-like
LSC7	Cysteine protease	*Brassica napus*	Oryzain ϒ-like
See2	Cysteine protease	Maize	Vacuolar processing
SAG12	Cysteine protease	*Arabidopsis*	
LSC790	Cysteine protease	*B. napus*	
LSC760	Aspartic protease	*B. napus*	
UBC4	Ubiquitin carrier protein	*Nicotiana sylvestris*	
UBI7	Polyubiquitin	Potato	
RNS2	Ribonuclease	*Arabidopsis*	Senescing petals
MS	Malate synthase	Cucumber	
ICL	Isocitrate lyase	Cucumber	
pBPCK-7A	Phosphoenlpyruvate carboxykinase	Cucumber	Cotyledons
gMDH	NAD+-malate dehydrogenase	Cucumber	
LSC101	Fructose-1,6-bisphosphate aldolase	*B. napus*	
LSC540	Glyceraldehyde-3-phosphate dehydrogenase	*B. napus*	
See3	Pyruvate, orthophospate dikinase	Maize	
LSC2C$_2$13	Pyruvate, orthophospate dikinase	*B. napus*	
pTIP11	β-Galactosidase	Asparagus	Postharvest
PLD	Phospholipase D	Castor bean	Detached leaf
Atgsr2	Glutamine synthetase	*Arabidopsis*	
	Glutamine synthetase	Radish	
	Glutamine synthetase	Rice	
LSC460	Glutamine synthetase	*B. napus*	
pTIP12	Asparagine synthetase	Asparagus	Postharvest
LSC54	Metallothionein I	*B. napus*	
JET12-like	Metallothionein	Sambucus	Leaf abscission
LSC2C$_2$10	Metallothionein II	*B. napus*	
rgMT	Metallothionein II	Rice	Stress-induced
LSC30	Ferritin	*B. napus*	
LSC680	ATP sulfurylase	*B. napus*	
GSTII-27	Glutathione *S*-transferase	Maize	
LSC650	Catalase	*B. napus*	
LSC550	Cytochrome P450	*B. napus*	
LSC2C$_2$60	Cytochrome P450	*B. napus*	
LSC2C$_2$26	Cytochrome P450	*B. napus*	
LSC94	PR1a	*B. napus*	
LSC2C$_2$22	Chitinase	*B. napus*	
LSC2C$_2$12	Antifungal protein	*B. napus*	
TOM13	ACC oxidase	Tomato	Fruit ripening
TOM75	MIP membrane channel	Tomato	Fruit ripening, water stress
LSC8	NADH:ubiquinone oxidoreductase	*B. napus*	

[1]Indicates type of enzyme or any other situations where induced expression of the gene has been detected.
PR, pathogensis; ACC, 1-aminocyclopropane-1-carboxylic acid; MIP, major intrinsic protein.

CHAPTER I

The darkness-induced and senescence-associated gene, *Ntdin*, encodes a protein functioning in molybdenum cofactor biosynthesis in tobacco plants

Introduction

As described in general introduction, senescence is a highly controlled process (Noodén et al., 1997; Smart, 1994) and many genes that are up-regulated during natural leaf senescence have been isolated and characterized (Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997). Radish (*Raphanus sativus* L.) *din1* (referred to *Rsdin1* in this thesis) is an example, although initially it was reported as a darkness-inducible gene (Azumi and Watanabe, 1991). Its transcripts accumulate under senescence-inducing conditions such as ethylene treatment and are repressed by senescence-retarding phytohormones such as cytokinins (Azumi and Watanabe, 1991). *Arabidopsis thaliana sen1* (referred to *Atsen1* in this thesis), a counterpart of *Rsdin1*, has also been shown to be darkness-inducible and associated with naturally and artificially induced senescence (Oh et al., 1996). The product of *Rsdin1* can be imported into isolated chloroplasts after being processed to a small mature form, which shows sequence similarity to the sulfide dehydrogenase from *Wolinella succinogenes* (Shimada et al., 1998). It was proposed that Rsdin1 is involved in sulfur metabolism of chloroplasts, although the exact functions of Rsdin1 and Atsen1 remain unknown.

Most molybdenum-containing enzymes contain a common cofactor, Moco. This cofactor is essential for the activity of several key enzymes like NR, XDH, sulfite oxidase, and aldehyde oxidase, which catalyze basic reactions in nitrogen, sulfur and carbon metabolism (Kisker et al., 1997; Zimmer and Mendel, 1999). The most of our knowledge on Moco biosynthesis has been obtained from analyses of mutants of *Escherichia coli*, in which 5 Moco-specific operons have been identified and designated *moa*, *mob*, *mod*, *moe*, and *mog*,

with involvement of more than 15 genes (Rajagopalan, 1996; Mendel, 1997). The biosynthesis of Moco requires multistep synthesis of MPT moieties followed by transfer of molybdenum (Rajagopalan, 1996; Schwarz et al., 2000). In plants, Moco-deficient mutants have been classified into six *cnx* (*c*ofactor for *n*itrate reductase and *x*anthine dehydrogenase) complementation groups, indicating that at least 6 genes participate in Moco biosynthesis (Mendel, 1997; Zimmer and Mendel, 1999).

In this Chapter, I describe isolation of tobacco *din* (*Ntdin*), whose expression is induced during senescence and also by sulfate and nitrate but not by phosphate. Using tobacco plants expressing *Ntdin* gene in antisense, I provide direct evidence that Ntdin functions in Moco biosynthesis.

Materials and Methods

Plant material and various treatments

Tobacco seeds (*N. tabacum* cv. Xanthi nc) were surface sterilized for 10 min in a 1% NaClO solution containing 0.1% Tween-20, washed several times in sterile water and placed on 0.5% agar containing 1/2 strength Murashige and Skoog (MS) medium in plastic containers. The plants were grown to their 8th leaf stage in continuous white light (90 µE) at 25°C unless stated otherwise. For treatments directed to roots, the agar was carefully removed and the plants were transferred to liquid 1/2 strength MS medium and maintained for another 2 days under the same conditions before starting the following treatments. Plants were transferred from 1/2 strength MS medium to 1/2 strength MS medium lacking either NO_3^-, SO_4^{2-} or PO_4^{3-}. In further experiments tobacco plants were transferred to water, nitrate- (19.4 mM KNO_3), sulfate- (150 µM $MgSO_4$) and phosphate- (125 µM K_2HPO_4/KH_2PO_4) containing solutions, respectively. The concentrations were equivalent to those in 1/2 strength MS medium. In case of hormone treatments, abscisic acid (ABA, 20 µM), 6-benzylaminopurine (BA, 20 µM), (-) jasmonic acid (JA, 20 µM) or indole-3-acetic acid (IAA, 20 µM) were added to the 1/2 strength MS medium. For ethylene treatment, plants were exposed to 10 µl/l ethylene gas in

special glass containers. A each time point, 2nd and 3rd leaves from the tops of two plants were harvested, frozen in liquid nitrogen and stored at -80°C until use. Leaves of older age were harvested from plants grown in soil under continuous white light (90 μE) at 25°C.

cDNA isolation, TAIL-PCR and DNA sequencing

Poly (A^+) RNA was isolated using QuickPrep mRNA Purification kit (Pharmacia) from total RNA prepared by AGPC method and converted into a λ Uni-ZAP (Stratagene) phage cDNA library. This library was screened with ^{32}P labelled-*Rsdin1* probe. A TAIL-PCR was performed by an original protocol (Liu et al., 1993) using the following three *Ntdin*-specific primers, din-R2 (5'-CATATCCTCCAGCAATGTCAGTCACCCC-3', complementary to nucleotides 596 to 569), din-R3 (5'-CACTCGTAGCCATAAATGATCTCTTCCCC-3', complementary to nucleotides 545 to 517), din-R4 (5'-GGAATGTTTATGGCCCCAGGAGC ATGCCC-3', complementary to nucleotides 411 to 383 in Fig. 3A) and an arbitrary primer [5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3'] on tobacco genomic DNA. Double-stranded plasmids were sequenced with ABI PRISM 310 sequencer using a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer, Norwalk, CT, U.S.A.) or with Hitachi SQ-5500 sequencer using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham) (Sanger et al., 1977). Sequence data were assembled and analyzed with the SDC-GENETYX genetic information processing program (Lipman and Pearson 1985; Software Development Co., Tokyo) and compared with sequences in non-redundant databases by BLAST program on network servers (Altschul et al., 1990).

Primer extension analysis

The Texas Red-labelled primer (5'-TGTCACTGATGATACATTCCCATACGTC-3') (provided by Nippon Flour Mills Co. Ltd., Atsugi, Japan), complementary to nucleotides 169 to 142 of Fig. 3A, was hybridized at 60°C for 1h with 10 μg of heat-denatured total RNA isolated by AGPC method from senescing tobacco leaves. The annealed primer was extended with a reverse transcriptase (Superscript II, GIBCO-BRL) in 30 μl of a reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM each dNTPs, and 10 mM DTT) at

42°C for 1h. The extended product was resolved by electrophoresis on a gel consisted of 6% Long Ranger (Pharmacia)/6.1M urea in parallel with the sequencing ladders of the *Ntdin* fragment obtained by a TAIL-PCR starting with the same Texas Red-labelled primer.

Genomic DNA isolation and southern hybridization

Tobacco genome DNA was isolated by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Restriction digests of 4 μg of DNA were separated by electrophoresis on 0.7% agarose gel. The gel was successively subjected to denaturation in 0.5 M NaOH containing 1.5 M NaCl and then was neutralization with 1 M Tris (pH 7.5) containing 1.5 M NaCl. After blotting onto a nylon membrane (Hybond N, Amersham) by capillary action in 20 x SSC. The DNA was crosslinked onto the membrane using a UV cross linker. The membrane was then subjected to hybridization in buffer containing 0.5 M Church phosphate buffer, 0.5 M EDTA (pH 8.0), and 7% SDS at 65°C for 16 h (Church and Gilbert, 1984). After washing three times in 40 mM Church phosphate buffer and 1% SDS at 65°C for 30 min, the membrane was autoradiographed with X-ray film.

RNA isolation and northern hybridization

Total RNA was isolated according to the ATA method (Nagy et al., 1988), or by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Total RNA (20 μg each) was separated by electrophoresis in formaldehyde-1.2% agarose gel and blotted onto a Hybond N membrane (Amersham) in 20 x SSC by capillary blotting. After crosslinking the RNA by UV irradiation, hybridization was carried out at 42°C for 16 h with gentle shaking in hybridization buffer containing ^{32}P-labelled cDNA probe, 50% (v/v) formamide, 10% dextran sulfate, 1x Denhardt's solution (0.02% (w/v) Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% (w/v) BSA), 0.5% (w/v) SDS, 3x SSC, and 50 mM Tris-HCl (pH7.5). The membrane was washed three times with gentle shaking in a solution containing 1x SSC and 0.1% SDS at 65°C for 30 min, and then autoradiographed with X-ray film.

Measurement of chlorophyll content

Using a plastic homogenizer cooled in liquid nitrogen, frozen plant tissue (from the same samples used for RNA extraction) was crushed to a fine powder in 1.5-mL microfuge tubes followed by the addition of 1.2-mL aliquots of 80% (v/v) acetone. Samples were vortexed for 15 sec and incubated in the dark at room temperature for 30 min with inversion every 10 min. Bleached plant material was removed by centrifugation (5 min; 10,000g) and 1-mL aliquots of supernatants transferred to new tubes. Chlorophyll a + b content of extracts was determined spectrophotometrically (Lichtenthaler, 1987). Chlorophyll content is presented relative to the control of the youngest leaf in a given experiment.

Construction of GFP fusion plasmid and fluorescence micropcopy

The *Ntdin* cDNA was amplified using the primer A (5'-TCACAAtcTAGAATTGCAATCAG-3', corresponding to nucleotides 56 to 79 in Fig. 3A except the lower case letters) and the primer B (5'-TCTAAATAACGggatCCAGCTTGG-3', complementary to nucleotides 354 to 331 in Fig. 3A except the lower case letters), and pNtdinRT as a template. In those primers *Xba*I and *Bam*HI sites (underlined) were introduced for cloning purpose, respectively. The amplified fragment was cloned into pGEM-T Easy vector (Promega) and the insert was confirmed by DNA sequencing. The plasmid was digested with *Xba*I and *Bam*HI and the 390 bp fragment, which covers the corresponding region of aa positions 1-93 of Ntdin protein, was inserted into *Xba*I-*Bam*HI cleaved pGFP2' vector [A unique *Kpn*I site of the original plasmid (pGFP2, provided by Drs. N.-H. Chua and Spielhofer) was changed into a *Bam*HI site, resulting in pGFP2' to create pNtdin(1-93)::GFP. This plasmid encodes a fusion protein with a truncated Ntdin at the N-terminal portion and GFP at the C-terminal portion. *A. thaliana* cauline leaves were biolistically bombarded, as described (Sheen et al., 1995), with gold particle (Bio-Rad) coated with the control plasmid pGFP2 or pNtdin(1-93)::GFP. After 6 h incubation at room temperature in complete darkness, the cauline leaves were viewed using a microscope (OLYMPUS PROVIS AX70) equipped with a fluorescence module.

Preparation of protein extracts and western analysis

To extract native proteins all of the following procedures were carried out several treatments.

The frozen plant material was ground with a mortar and a pestle under liquid nitrogen. After transfer to a 1.5-mL microfuge tube the powder was homogenized by vortexing in extraction buffer [50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, 5 μg/ml aprotin, 5 μg/ml leupeptin and 10% (v/v) glycerol]. The homogenates were centrifuged at 14,000 x g and aliquots of the supernatants were frozen in liquid nitrogen and stored at -80°C. The protein concentrations were determined using a protein assay kit (Bio-Rad) based on the method of Bradford (1976), with bovine serum albumin as the standard. Western analysis was performed as described (Berberich et al., 1999). The most C-terminal region of Ntdin, NH_2-(C)AAWTENGLPTDS-COOH, was synthesized. This peptide was coupled to keyhole limpet hemocyanin and the conjugate was injected into rabbits to raise anti-Ntdin antibodies, which were affinity-purified. Equal amounts of protein were fractionated on SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984). Non-specific binding sites were blocked by incubating the membrane in PBS containg 5% non-fat-dry milk. Ntdin protein was identified in the blots by a chemiluminescence method (Amersham) according to the manufacturer's instructions, using a 1:1000 dilution of the affinity-purified anti-Ntdin antibody as the 1st antibody.

Tobacco transformants expressing Ntdin *in antisense*

The *Ntdin* cDNA fragment sandwiched with *Sac*I and *Xba*I sites was subcloned into the respective sites of pBI121 (Clontech) to create pBI-antiNtdin. As a control, pBI121 was used for *Agrobacterium*-mediated transformation. Tobacco (*N. tabacum* cv. Xanthi) leaves were co-cultured with the resulting transformants and transgenic plants were selected on MS agar containing 50 $\mu g/ml^{-1}$ Kanamycin. The presence of a transgene in the plants was confirmed by genomic PCR with appropriate primers.

Chlorate sensitivity test

Transgenic tobacco plants were cut at the bottom of their stems and aseptically transferred to 1/2 strength MS medium containing 3 mM or 5 mM potassium chlorate. After 4 weeks of

culture, phenotype and root growth were assayed.

Determination of nitrate reductase activity

NR (NADH: nitrate oxidoreductase, EC1.6.6.1) activity was measured in crude extracts as described by Scheibel et al. (1997). The plant meterial stored in liquid nitrogen was ground to a fine power in a mortar that had been precooled with liquid nitrogen. The powered material was thoroughtly mixed and extracted with four leaves nolumes (v/w) of ice-cold buffer [100 mM HEPES-KOH (pH 7.5), 5 mM $Mg(OAc)_2$, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 1% (w/v) BSA, 0.1% Triton X-100, 0.5 mM phenylmetylsulphonyl fluoride (PMSF), 20 µM FAD, 25 µM leupeptin, 5 µM Na_2MoO_4, 1% polyvinylpyrolidone (PVPP)]. NR activity was measured immediately by mixing one volume of extract with five volumes of prewarmed (25°C) assay buffer [100 mM HEPES-KOH (pH7.5), 5 mM KNO_3, 5 mM EDTA (pH7.5), 0.25 mM NADH]. After various times at 25°C, 300 µl aliquots were reved from the assay mixture and the reaction was stopped by adding 25 µl 0.6M zinc acetate and 75 µl 150 µM phenazine methosulphate, mixed and held for 15 min before adding 300 µl 1% (w/v) sulphanilamide in 3N HCl + 300 µl 0.02% (w/v) *N*-(1-naphtyl)-ethylenediamine in bidestilled water. Tubes were allowed to stand for 20 min at room temperature, centrifuged at 14,000 x g and the absorbance of the produced azo-dye in the clear supernatant was measured at 540 nm.

Determination of xanthine dehydrogenase activity

Native gel electrophoresis and detection of XDH activity was as described by Mendel and Müller (1976). The plants were watered with a 10 mM KNO_3 solution 12 h before harvesting the leaves. Crude extracts were prepared by grinding the tissue in a chilled mortar with acid-wased quartz sand and two times its weight (w/v) of cold 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 1% triton X-100. The slurry was centrifuged at 4°C for 30 min at about 20,000 x g and the supernatant was taken for enzyme electrophoresis. Electrophoresis under non-denaturing coditions was carried out in 7% polyacrylamide gel (ratio of N,N' methylene-bis-acrylamide to acrylamide 0.026). A 2.25% spacer gel (ratio of N,N' methylene-bis-acrylamide to acrylamide 0.25) was

polymerized on top of the separating gel. The buffer in the reservoirs was 0.05 M TBE buffer, pH 8.3. After adding bromophenol blue as tracking dye and sucrose, electrophoresis was carried out at 5 mA per gel (anode on the bottom), until the tracking dye reached the bottom of the gel column. After electrophoretic separation the gels were equilibrated in 0.1 M sodium pyrophosphate buffer, pH 8.0, and transferred to the following staining mixture: 0.1 M sodium pyrophosphate buffer, pH 8.0, containing 2 mM hypoxanthine, 1 mM nitrotetrazolium blue, 0.1 mM phenazine methosulphate at 30°C in the dark for 30 to 60 min.

Determination of Moco content

The assay for Moco was based on the determination of the activity of NADPH-nitrate reductase (EC 1.6.6.3) assembled with NR apoprotein of the Moco-deficient *nit-1* mutant of *Neurospora crassa* and a Moco fraction of tobacco leaves according to Mendel et al. (Mendel et al., 1981, Mendel et al., 1985). L929 and NR6P cells obtained from a 6-cm dish were homogenized by sonication in 200 µl 50 mM potassium phosphate, 5 mM EDTA, and 5 mM reduced glutathione (pH 7.2). After clarification, 200 µl of protein extract were immediately transferred to 50 µl of *nit-1* extract supplemented with 20 mM NADPH and complemented anaerobically overnight at 4°C. Reconstituted NADPH-nitrate reductase activity was assayed as described (Stallmeyer et al., 1999).

Results

Isolation Ntdin *cDNA and* Ntdin *5' upstream TAIL-PCR fragment*

Using the Radish *din1* as a probe (provided by Prof. A. Watanabe, Univ. of Tokyo), two positive clones were obtained from the λ Uni-ZAP phage cDNA library ($2x10^4$ pfu) derived from senescing tobacco leaves. Both the clones were originated from a gene and the longer cDNA insert was ca. 0.7 kb. It seems not to be a full-length cDNA with nucleotide sequence comparison of the *Rsdin1* and *Atsen1* cDNAs (Azumi and Watanabe, 1991, Oh et al., 1996, Shimada et al., 1998). To obtain a sequence information on the full length cDNA

and the further 5' upstream region, the TAIL-PCR with three gene's specific primers (1st cycle, dinR2 and the arbitrary primer described; 2nd cycle, dinR3 and the arbitrary primer then 3rd cycle, dinR4 and the arbitrary primer) on tobacco genomic DNA as a template were performed. The amplified 845 bp sequence was seemed to contain two introns, 89 bp (1st) and 105 bp (2nd). In Fig. 3A, these intron sequences were omitted and their positions were indicated by vertical arrowheads. Based on the sequence, the two primers, (5'-CAAAGAGCTGATCATCTTTTCAAAATCTCACTC-3', corresponding to nucleotides 6 to 38) and (5'-CATCAGAATGAGGAGAACATTTACACATATGG-3', complementary to nucleotides 868 to 837), were designed and a RT-PCR on total RNA prepared from senescing tobacco leaves was performed. Due to direct sequencing of the 863 bp-amplified fragment, the DNA sequence of the cDNA, termed *Ntdin* (standing for *N.tabacum din1* homologue), was confirmed. This fragment was also cloned into pGEM-T Easy vector (Promega), resulting in the plasmid pNtdinRT. *Ntdin* cDNA shows 56.2% and 55.5% identity to *Atsen1* cDNA and *Rsdin1* cDNA, respectively. *Ntdin* cDNA contains an ORF of 558 bp encoding a 185 amino acid-protein (named Ntdin) with a relative molecular mass of 20,095 daltons. Its inferred amino acid sequence has 56.8% and 54.2% identity to the *Arabidopsis* protein (Atsen1) and the radish one (Rsdin1), respectively (Fig. 3B). Their N-terminal regions (1 to 71 amino acid positions in case of Ntdin) are fairly diverse and the residual C-terminal halves (amino acid positions 72 to 185) shows 68.1% and 69.0% identity to the corresponding regions of Atsen1 and Rsdin1, respectively. This observation suggests that these diversed regions are signal peptides and the processed C-terminal regions may be targeted to organella. A primer extension analysis revealed that the transcriptional start site of *Ntdin* was the nucleotide T located 76 bp upstream of the first nucleotide of translational start codon (Fig. 4). A putative TATA box, TAAATAT, was located in –40 to –34 (Fig. 3A). Two Myc recognition sites (CANNTG, Murre et al., 1989) (–207 to –202 and –91 to –86) and a single Myb recognition site (C/TAACNA/G, Bíedenkapp et al., 1988; Nakagoshi et al., 1990) (-192 to –187) were observed in this region. A sequence, 5'-GCTGACGTG-3', which was similar to ABA-responsive element (Marcotte et al., 1989) and totally conserved in both *Rsdin1* and *Atsen1* promoter sequence and located ca. 40 bp upstream of their TATA boxes [(Oh et al.,

1996, *Rsdin1* genome sequence (Accession # D38252)], was also observed in *Ntdin* promoter region, 5'(-125)-CTTTACGTG-3'(-117) (bold letters represent conserved nucleotides in the tobacco sequence), although its significance was unclear at this moment.

Genomic southern analysis

Two or three hybridization signals per lane were detected in genomic Southern hybridization using *Ntdin* ORF fragment as a probe (Fig. 5). It indicates that *N. tabacum* genome DNA contains at least two copies of *Ntdin* genes.

Tissue specific accumulation of Ntdin *mRNA in mature tobacco plant*

The effects of leaf senescence on *Ntdin* expression were investigated by northern hybridization. The mRNA level of *rbcS* was well correlated with the total chlorophyll content (Fig. 6A). The steady-state levels of *Ntdin* mRNA had reverse correlation with them. In young flower bud and root *Ntdin* mRNA was detected at very low level and at scarce level, respectively (Fig. 6B).

Expression of Ntdin *on the mRNA level in response to various treatments*

With the use of northern hybridization, overall changes in the mRNA level of *Ntdin* after various treatments of tobacco plants were investigated (Fig. 7). The *Ntdin* mRNA accumulated continuously during dark treatment in the leaf tissue within 24 h (Fig. 7A). A prolonged exposure to dark (2d and 4d) caused a slight drop of the mRNA level to the same level as after 12h in the dark indicating a maximum after 24h. Because senescence changed nitrogen, sulfur and phosphorus levels, the effects of deprivation and addition of these elements on *Ntdin* expression were addressed. Withdrawal of nitrate, phosphate or sulfate from 1/2 strength MS medium over a period of 3 or 6 days did not cause any change (Fig. 7B). Application of phosphate for the same periods also did not cause any change. In contrast, nitrate and sulfate elevated the levels of *Ntdin* mRNA (Fig. 7B). Addition of glutamine, cysteine and thiosulfate also resulted in *Ntdin* mRNA accumulation (data not shown). Since leaf senescence is affected by phytohormones, their effects on *Ntdin* expression were

analyzed. After ethylene (1d and 2d) treatment the *Ntdin* transcript levels increased in leaves to approximately the same extent as after 6h darkness. ABA and IAA had a profound effect on *Ntdin* expression (Fig. 7C), whereas BA and JA had no influence (Fig. 7C). The results suggest that Ntdin is involved in both nitrogen and sulfur metabolism and that it's expression is regulated by phytohormones.

Change in the level of Ntdin protein under various growth conditions

With anti-Ntdin antibody, three proteins were detected in leaves upon darkness in western analysis. The 16.4 kDa protein was found non-specifically in young seedlings. Two other proteins of 15.8 kDa (protein a) and 14 kDa (protein b) accumulated upon darkness (Fig. 8A), their amounts being increased at 24h by ABA- and IAA- but not BA- and JA-treatments (Fig. 8A). The amounts of Ntdin was consistent with changes in level of *Ntdin* transcripts. Upon ethylene-treatment, however, proteins a and b were predominant whereas *Ntdin* transcripts were detected at only low level. In mature tobacco plants, proteins a and b were consistently detected regardless of aging, as judged from chlorophyll contents (Fig. 8B).

Similarity of Ntdin with C-terminal region of Cnx5

A homology search revealed that Ntdin shows considerable similarity to the C-terminal regions of several MoeB (MPT synthase sulfurylase) and Cnx5 (MoeB homolog in plant) proteins (Fig. 9A and 9B). For this paper, the N-terminal (about 240 amino acids) and the C-distal (about 90 amino acids) regions of Cnx5 in *N. plumbaginifolia* were tentatively termed domains A and B, respectively. Ntdin scores 25% identity to the domain B of *N. plumbaginifolia* Cnx5 (Fig. 9B). Even in the prokaryote *Mycobacterium tuberculosis* MoeB retains the domain B, but not in *E. coli* and *Bacillus subtilis*.

Cellular Localization of the Ntdin Protein

Amino acid alignment of Ntdin, Atsen1 and Rsdin1 suggested that the region constituted by amino acid positions 1 to 71 of Ntdin functions as a signal peptide and that the processed C-terminal conserved regions may be targeted to organelles. To identify a cellular

localization of Ntdin protein, we constructed a cauliflower mosaic virus (CaMV) 35S promoter::*Ntdin-GFP* gene fusion that could be used in transient assays. After biolistic bombardment into *A.thaliana* cauline leaves, the GFP signal from the control plasmid (pGFP2) was observed in the cytoplasm and nucleus (Fig. 10A). In contrast, the Ntdin(1-93)::GFP fusion protein is exclusively in the chloroplasts, indicating that Ntdin localizes to the chloroplasts (Fig. 10B).

Chlorate sensitivity in Ntdin-*repressed tobacco plants*

Since *Ntdin* mRNA accumulated on application of nitrate or sulfate but not phosphate, a common role in nitrogen and sulfur metabolism was suggested. The similarity of Ntdin with the domain B of Cnx5 proteins further indicated an involvement in molybdenium metabolism. Moco is essential for NR, which is the key enzyme of nitrogen metabolism, and for sulfite oxidase, which may be the key enzyme of sulfur detoxication. To examine this possibility, transgenic tobacco plants were generated, in which expression of endogenous *Ntdin* gene(s) was suppressed by the antisense method. Since chlorate is a toxic nitrate analog that has been used to screen for mutants defective in Moco biosynthesis, transgenic plants were assayed for $KClO_3$-sensitivity. Thirty-four to 38 independent transgenic plants, in which the presence of the transgene was confirmed by genomic DNA-PCR, were randomly selected, cut off at the bottom of stems, transferred into 3 or 5 mM $KClO_3$-containing medium and further incubated at 25°C. After 4 weeks, root growth was compared with that of controls. Of the *Ntdin*-antisense transgenics 74% and 32% generated roots in 3 mM- and 5 mM-$KClO_3$ media, respectively, in contrast to 44% and 6% of control transgenics (Table 2). AntiNtdin transgenic plants also grew far better than controls in chlorate-containing media (Fig. 11).

Ntdin protein level, NR and XDH activities and Moco contents in transgenic plants

Four lines of chlorate-resistant antiNtdin transgenic plants were further analyzed and shown to contain a scarcely any amounts of Ntdin proteins than wild type tobacco plants (Fig. 12A). They showed approximately 7-8% NR activity of that of wild type plant (Fig. 12B) and exhibited fairy lower XDH activities (Fig. 12C) compared to the control plants. Measurement

using *N. crassa nit-1* cell extracts revealed that they contained less than 5% of Moco compared with the controls (Fig. 12D).

Discussion

This chapter documents evidence that Ntdin functions in Moco biosynthesis, which may be required during leaf senescence. Transgenic tobacco plants with reduced Ntdin activity showed scarce NR activities and lower XDH activities and chlorate tolerance, similar to the phenotype of Moco-deficient plant mutants (Stallmeyer et al., 1999). Direct measurement of Moco contents revealed that it was the case in the Ntdin antisense plants. Aldehyde oxidase, another molybdoenzyme, has recently been shown to catalyze the last step in the biosynthesis of the phytohormones IAA (Koshiba et al., 1996) and ABA (Walker-Simmons et al., 1989; Xiong et al., 2001). *Ntdin* transcripts here specifically accumulated with exogenous application of these two hormones, implying that end products catalyzed by aldehyde oxidase modulate the expression of *Ntdin*.

The level of Ntdin protein correlates with the level of *Ntdin* transcripts, although there were some exceptions with cases of ethylene treatment and senescing leaves. The results thus suggest that the abundance of Ntdin is mainly controlled at the transcriptional level, and to a lesser extent, at the post-transcriptional level. Detection of two proteins of different sizes by anti-Ntdin antibody can possibly be explained as follows: First, one of the proteins is the product of a second copy *Ntdin* gene. Genomic Southern hybridization using the *Ntdin* ORF fragment as a probe showed two or three hybridization signals per lane, supporting the presence of at least two copies of the *Ntdin* gene per haploid genome of *N. tabacum* (Fig. 5). Second, the products from the two *Ntdin* genes are the same in size but processed differently. Isolation of the second *Ntdin* gene is necessary to determine the mechanism.

Ntdin seems to be a monofunctional protein, whereas *N. plumbaginifolia* Cnx5 is a two-domain protein, comprising *E.coli* MoeB and a domain B. Some of Moco biosynthetic proteins in eukaryotes are known to be multifunctional proteins. For example, *Arabidopsis*

Cnx1 (Stallmeyer et al., 1995) and mammalian Gephyrin (Stallmeyer et al., 1999) consist of *E. coli* MogA and MoeA. The functional relationship between the domain B of Cnx5 and Ntdin remains to be clarified. To identify an interacting protein(s) with Ntdin may help to understand its authentic function.

Ntdin was here found to be localized in chloroplasts, suggesting that certain steps of Moco biosynthesis in plants occur in these organelles. In a current model for the Moco biosynthesis pathway in plants (Zimmer and Mendel, 1999), the first step is the conversion of a guanosine derivative into a precursor Z, catalyzed by the *cnx2* and *cnx3* gene products which are homologous to MoaA and MoaC of *E. coli*. *A. thaliana* Cnx2 and Cnx3 proteins have some of the characteristics of transit peptides for the chloroplasts and mitochondria (Hoff et al., 1995). Guanosine is made in plastids (Senekoff and Meagher, 1993). These data support the idea that at least some steps in Moco biosynthesis are plastid localized.

Which step of Moco biosynthesis is catalyzed by Ntdin is an unanswered question. Ntdin also has a similarity to an *A. thaliana* senescense-associated rhodanese-like protein (AAD03573) and to mammalian rhodaneses (Pallini et al., 1991; Dooley et al., 1995) (Fig. 13). Rhodanese (EC 2.8.1.1; thiosulfate sulfurtransferase), both in prokaryotes and eukaryotes, enzymatically inactivates cyanide (CN-) by converting it into less toxic thiocyanate. This reaction involves the transfer of a sulfur atom from a donor cofactor to the substrate. The catalytic intermediate of the enzyme takes a form containing the transferred sulfur atom. The sulfur has been suggested to exist in a persulfide linkage involving the active site sulfhydryl group, Cys-247, of rhodanese (Luo and Horowitz, 1994). This Cys residue in rhodanese is conserved in the corresponding positions of Ntdin, Atsen1 and Rsdin1 (Fig. 1A). The Arg residue (Arg-187) of rhodanase responsible for substrate binding is also conserved in Ntdin and Atsen1, and replaced with a similar positively charged Lys residue in Rsdin1. It is likely that tobacco Ntdin has sulfur transferase activity *per se* or in association with other subunits in chloroplasts, acting on unidentified Moco biosynthetic intermediate(s). This will be tested using *E. coli*-produced Ntdin protein in the future. Furthermore, determination of the structure of MPT and its derivatives in wild type and antiNtdin-transgenic tobacco plants should help define the function of Ntdin in Moco biosynthesis.

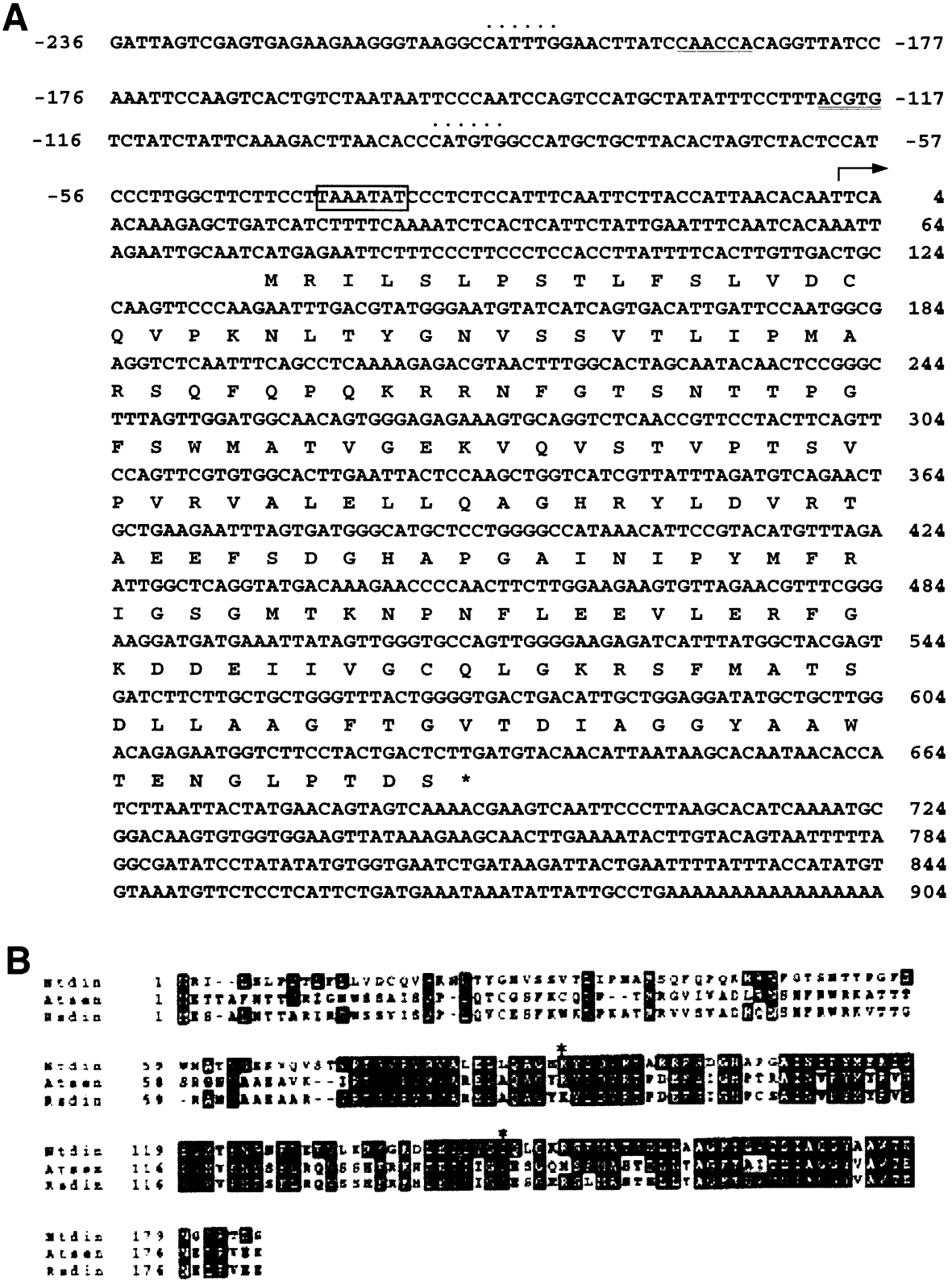


Fig. 3 (**A**) Nucleotide sequence and the deduced amino acid sequence of the *N. tabacum Ntdin* gene. (**B**) Amino acid sequence alignment of Ntdin, *Arabidopsis* Atsen1 and Radish Rsdin1 proteins. (**A**) Numerals are started from a transcription start site (indicated by a right-angled arrow, see Fig. 4). A putative TATA box is squared. Myc recognition sites (CANNTG) and Myb recognition site (C/TAACNA/G) are over-dotted and underlined, respectively. ABRE (A/GT/CACGTGGT/CA/G) is double-underlined. An asterisk indicates a stop codon. Two intron positions are indicated by vertical arrowheads. (**B**) Black boxes represent sequence identity, and light grey boxes represent sequence similarity in the alignment. Dashes were introduced to maximize the alignment.

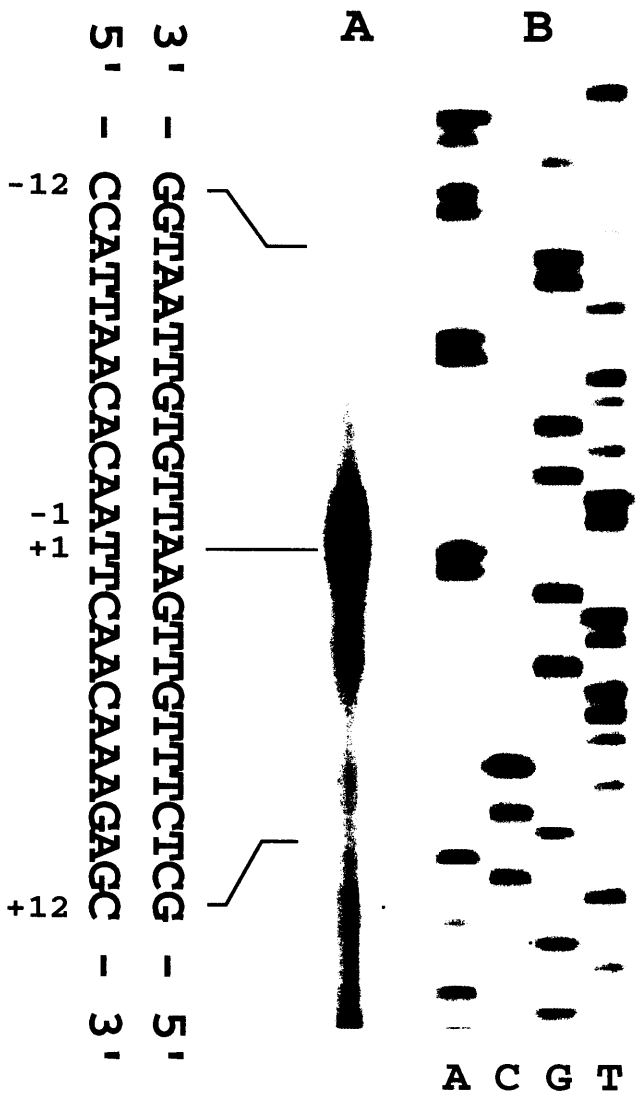


Fig. 4 A primer extension analysis to determine a transcriptional start site of *Ntdin* gene. A Texas-Red labelled primer (see Materials and Methods) was hybridized with 10 μg of total RNA from senescing leaves and extended by reverse transcriptase. Sequencing reaction for the TAIL-PCR fragment was carried out with the same labelled primer. The extended product (marked with A) was electrophoresed in parallel with the sequencing products (marked with B) on 6% Long Ranger/ 6.1 M urea gel. The major extended product was indicated by arrow.

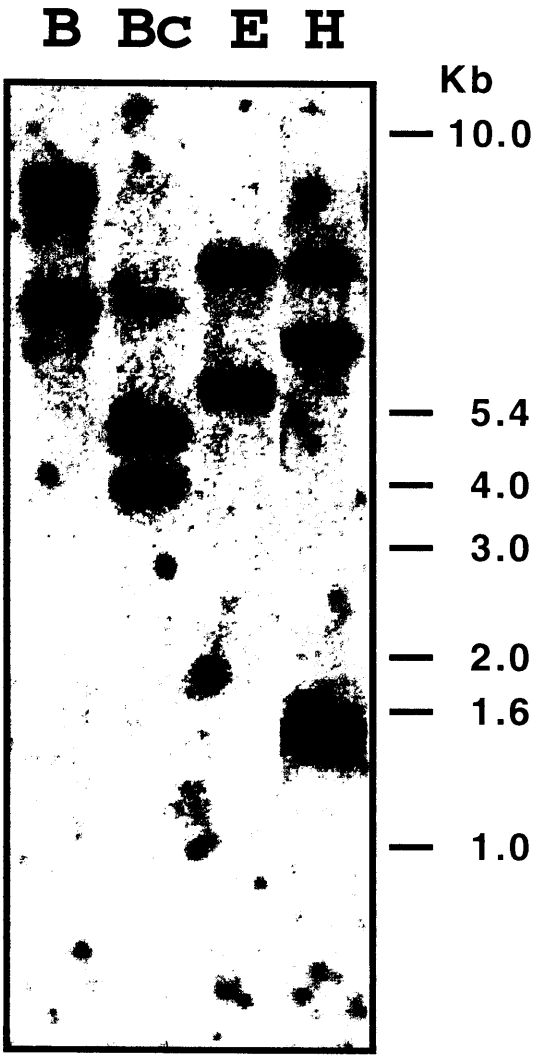


Fig. 5 Genomic Southern hybridization of *Ntdin*. Tobacco genome DNAs (10 μg each) digested with *Bam*HI (B), *Bcl*I (Bc), *Eco*RI (E) and *Hin*dIII (H) were sized-separated by a 0.7% agarose/TBE electrophoresis and capillary-blotted to Hybond-N+ (Amersham) membrane. After UV cross-linking, a membrane was probed with *Nidin* ORF fragment.

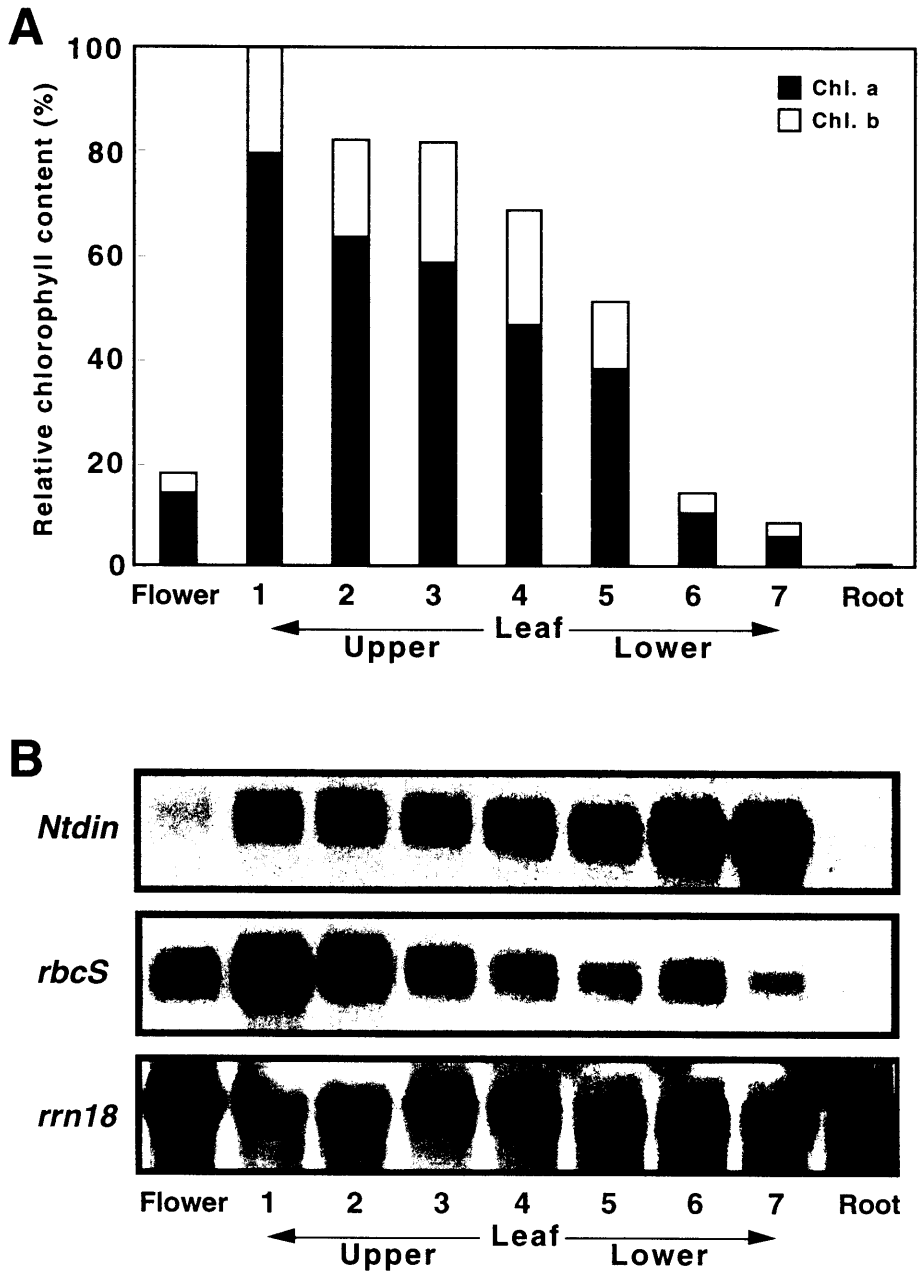


Fig. 6 Tissue specific expression of *Ntdin* in a mature tobacco plant. Tobacco plant was grown in a greenhouse. Various tissues including seven different positions of leaves, and young flower buds and root, of a tobacco plant were harvested at daytime of a sunny day and stored in liquid nitrogen until use. (**A**) Chlorophylls were measured as described in Materials and Methods. (**B**) Total RNAs were also extracted from the same tissues and the transcript levels of *Ntdin* and *rbcS* (provided by Dr. K. Tomizawa, RITE) were estimated by northern hybridization. To ensure the equall loading of the RNA samples, the blot was also hybridized with ribosomal 18S DNA (*rrn18*, Ganal and Hemleben, 1986).

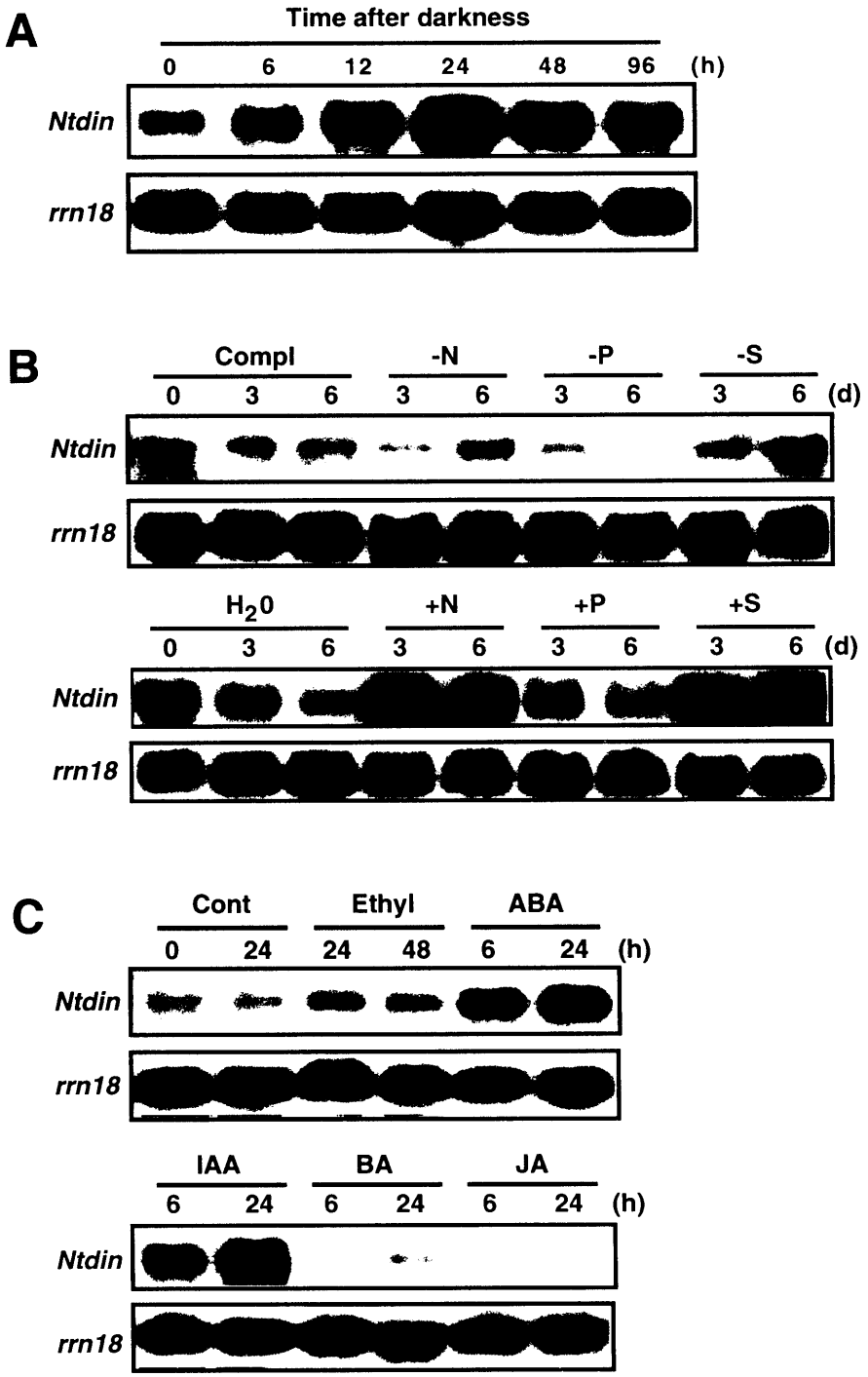


Fig. 7 Changes in the level of *Ntdin* transcripts in leaves of tobacco seedlings after various treatments. (**A-C**) Plants were grown and treated as described and total RNAs were extracted for northern blotting. (**A**) Plants were transferred from continuous light to the dark for the times indicated. (**B**) Plants were treated by withdrawal of nitrogen (-N), phosphorus (-P) or sulfur (-S), respectively, from the MS medium (compl). Plants were supplied by addition of nitrogen (+N), phosphorus (+P) or sulfur (+S). (**C**) Plants were treated with phytohormones for the indicated periods.

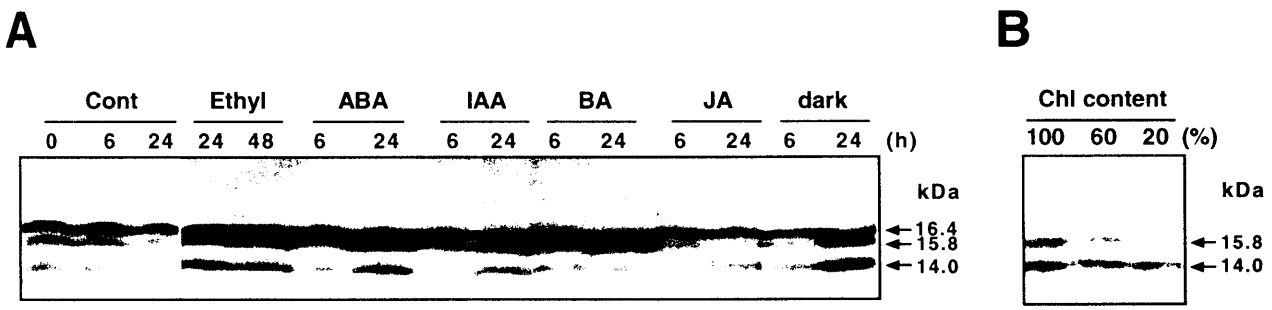
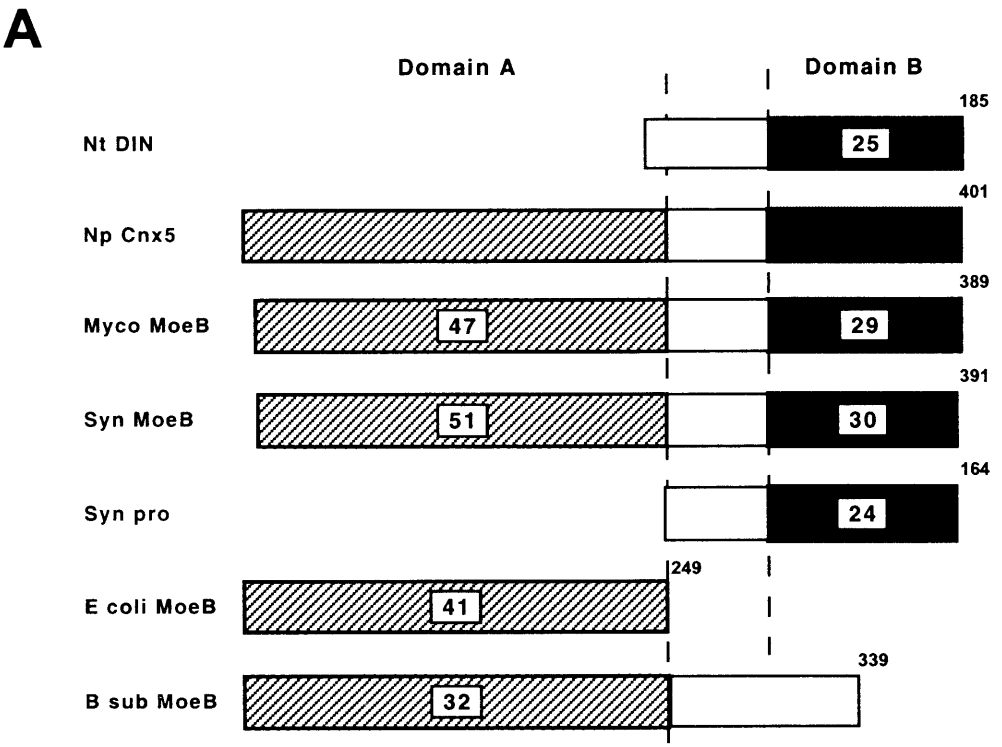


Fig. 8 Change in Ntdin levels in tobacco plants treated with phytohormones and exposed to darkness (**A**), and in leaves with different chlorophyll contents (**B**). Plants were grown and treated as described and equal amounts of extracted proteins (30 μg each) were separated for western blotting. The duration of the treatments is indicated. Relative molecular masses (in kDa) of the proteins were calculated using the SigmaGel program.



B

```
Ntdin        LDVRTAEEFSDGHAPGAINIPYMFRIGSGMTKNPNFLEEVL-E----RFGKDDEIIVGCQ  146
Synprotein   VDVREVKEYQAGHIPGAINIP-LRTLSHNLAQIP----------------PDRKVIFYCS  124
NpCnx5       VDVRPAHHYKIVSLPNSVNIP-LSTLEGRLAEISAALEKEADKET-FDSGSGASLHTICR  360
AtCnx5       LDVRPSHHYKIVSLPDSLNIP-LANLETRLNELTSALKEKGNGHANTESCTNPSVFVVCR  423
SynMoeB      IDVRNPHEAEIATIPGAILVP-LPDIEAGAGVETVRE----------LLNGKRELIVHCK  351
MtMoeB       VDVREPAEFDIAHIPGSILIP-KGEIGSAAGLA----------------QLPLDKEIVLYCK  348

Ntdin          GKRSFMATSDLLAAGFTGVTDIAGGYAAWTENGLPTDS--                    185
Synprotein   TGYRSAMAMMALNLLGYENVLAFSPSFTGWQAAGEAIAKA-                    164
NpCnx5       RGNDSQRAVQLLHKAGFTSAKDIIGGLESWAENVDPKFPTY                   401
AtCnx5       RGNDSQRAVQYLRESGFDSAKDIIGGLEAWAANVNPNFPTY                   464
SynMoeB      MGGRSAKALGILKEAGITGTN-VAGGINAWSQDVDPSLPQY                   391
MtMoeB       SGIRSAQALTTLKAAGLHNVKHLDGGIAEWTRTIDSSLLVY                   389
```

Fig. 9 (**A**) Schematic illustration of the similarity of Ntdin to the C-terminal regions of several Cnx5 (MoeB) proteins and a Synochosystis protein. (**B**) Amino acid alignment of Ntdin, a Synochosystis protein and tentative B domains of several Cnx5 (MoeB) proteins. Numerals along the sequences or in the right shoulders of the bars indicate the amino acid positions. (**B**) Black boxes indicate sequence identity, and light grey boxes sequence similarity in the alignment. Dashes were introduced to maximize the alignment. The residues discussed in the text are marked with asterisks. (**A**) Domains A and B are indicated by cross-stripes and dense-dots, respectively. Similarity (in per cent) to NpCnx5 A and B domains is given onto each domain-bar. (**B**) Synprotein (D90917), NpCnx5 (*N. plumbaginifolia*, AF124161), AtCnx5 (*A. thaliana*, AF124159), SynMoeB (Synechococcus PCC7942, Y16560), MtMoeB (*M. tuberculosis*, Z95150), E coli MoeB (*E. coli*, D90720) and B sub MoeB (*B. subtilis*, AF012285).

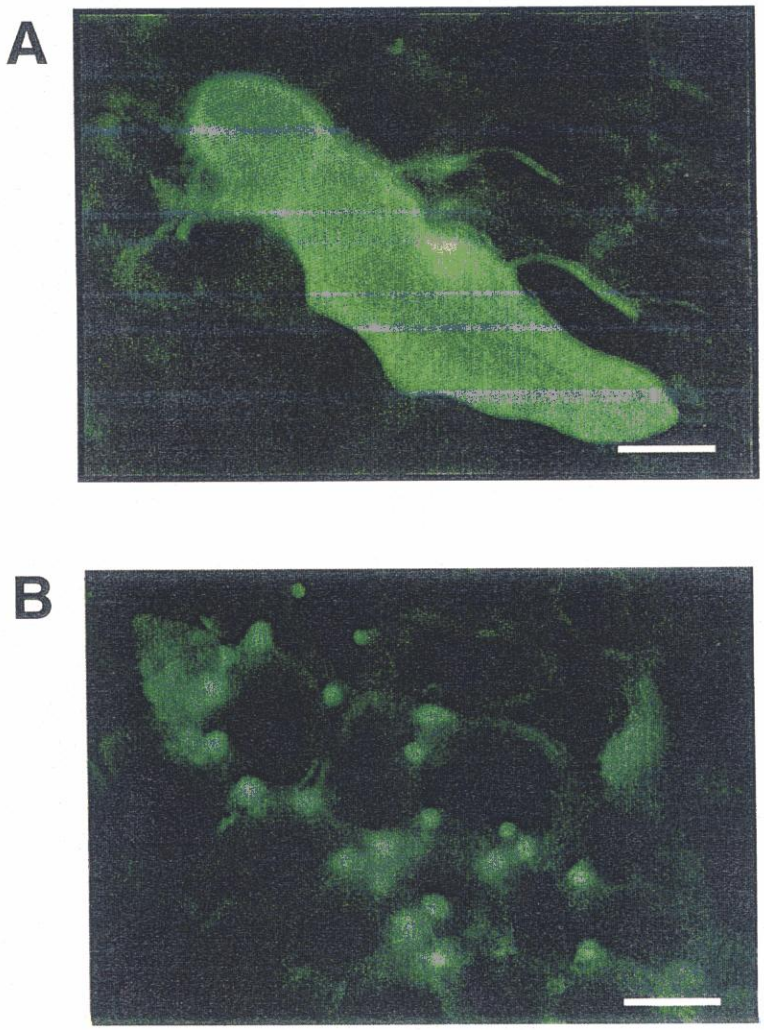


Fig. 10 Chloroplast localization of the Ntdin::GFP fusion protein in *A. thaliana* cauline leaves. *A. thaliana* leaves were bombarded with gold particles coated with (**A**) pGFP2 and (**B**) pNtdin::GFP plasmid. The proteins were transiently expressed and individual cells are shown under epifluorescence. Scale bars are for 200 μm.

Table 2 Chlorate sensitivity of the transgenic tobacco plants transformed by control vector (pBI121) and pBI-antiNtdin construct.

$KClO_3$ conc. (mM)	Transgenics transformed by	Rooting	
		-	+
3	pBI121	20/36 (56)	16/36 (44)
	pBI-antiNtdin	10/38 (26)	28/38 (74)
5	pBI121	32/34 (94)	2/34 (6)
	pBI-antiNtdin	24/35 (68)	11/35 (32)

*Digits in parentheses indicate per cent in total plants tested.

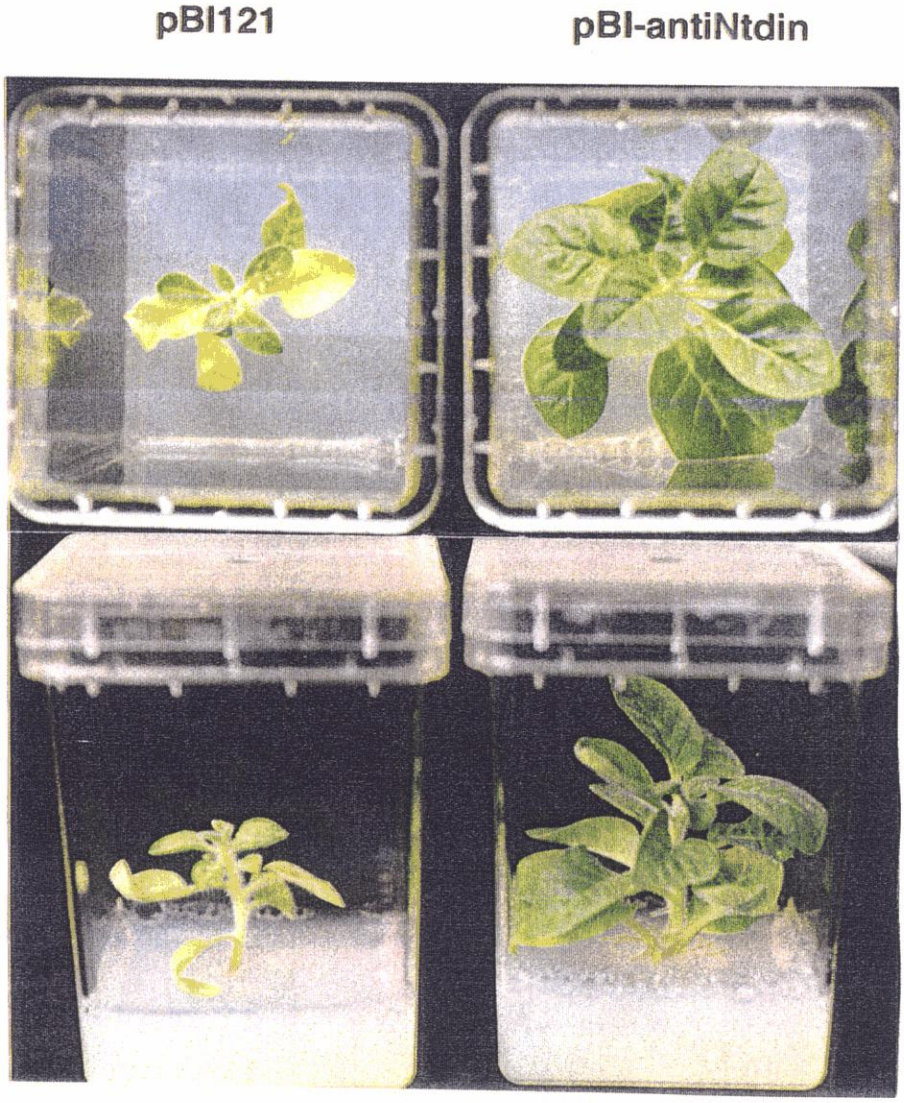


Fig. 11 Chlorate sensitivity of pBI121-transgenic and pBI-antiNtdin-transgenic plants. Representative phenotypes of pBI121- (left) and pBI-antiNtdin-transgenic (right) plants in 3 mM $KClO_3$-containing media are illustrated.

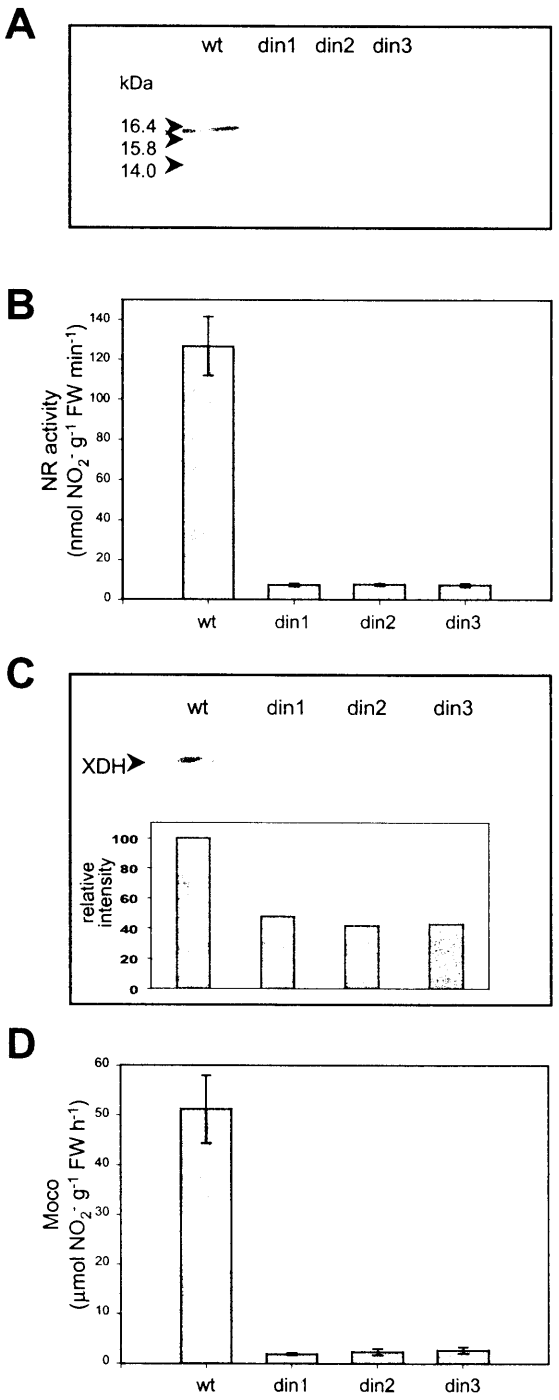


Fig. 12 Levels of Ntdin protein(s), XDH and NR enzyme activities and Moco contents in wild type and pBI-antiNtdin transgenic plants. (**A**) Detection of Ntdin protein(s) by western analysis. Leaves were harvested just before starting a light period. (**B**) NR activity, (**C**) XDH activities, assessed using hypoxanthine as the substrate, and (**D**) Moco content. The values (means±SE) were obtained from three independent experiments in duplicate assays. In **B** and **C**, leaves were harvested 4h after starting a light period.

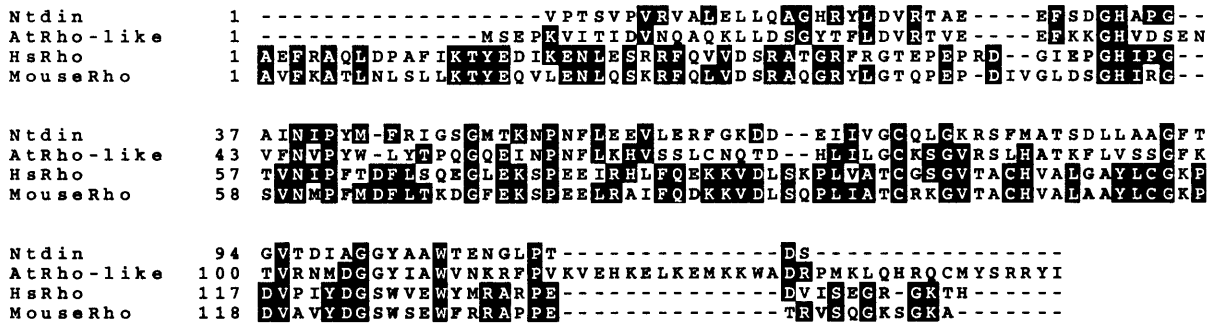


Fig.13 Amino acid alignment among Ntdin, *A. thaliana* rhodanese-like protein and human and mouse rhodaneses. The positions of the conserved Arg and Cys at the active site of mammalian rhodaneses (Ploegman et al., 1978; Luo and Horowitz, 1994) are marked by stars. The two other important residues for substrate recognition in rhodanese (Nagahara et al., 1995; Nagahara and Nishino, 1996) are indicated by black dots.

CHAPTER II

Specific association of transcripts of *tbzF* and *tbz17*, tobacco genes encoding bZIP-type transcriptional activators, with guard cells of senescing leaves and/or flowers

Introduction

In Chapter I, I described senescence-associated gene (*Ntdin*) from tobacco plants to be involved in molybdenum metabolism. In order to understand how these senescence-associated gene are switched on, I examined in this chapter, genes encoding bZIP transcriptional activators.

The control of transcription is mediated through recognition of *cis*-elements by transcription factors (Meshi and Iwabuchi, 1995; Yanagisawa, 1998), classified into several groups on the basis of structural motifs. One of the major families of transcription factors is constituted by the basic-region leucine-zipper (bZIP) proteins (Landschulz et al., 1988; Hurst, 1994). Genome analysis of Arabidopsis resulted in an estimate that this organism contains almost 100 bZIP-encoding genes (Riechmann and Ratcliffe, 2000).

Within the bZIP-gene family in plants, the *lip19* subfamily consists of rice *lip19* (Aguan et al., 1993), maize m*lip15* (Kusano et al., 1995) and *OBF1* (Singh et al., 1990), radish *rlip* (Ito et al., 1999), *910* and *911* from *Antirrhinum majus* (Martínez-García et al., 1998), and tobacco (*Nicotiana tabacum*) *tbz17* (Kusano et al., 1998). These are all characterized by upregulation of transcript levels upon exposure to low temperature. The *A. majus* genes, *910* and *911*, are expressed in flowers, predominantly in vascular tissues, carpels and anthers (Martínez-García et al., 1998). *OBF1* of maize was first identified as a gene encoding a binding factor to the *ocs*-element found in the promoter region of the octopine synthase gene of the *Agrobacterium tumefaciens* T-DNA (Singh et al., 1990) and demonstrates a gradient in transcript levels in developing leaves. In the basal portions, which contain dividing and

differentiating cells, the *OBF1* transcript level is 40-50 fold higher than in apical portions, where the cells are fully differentiated. This indicates developmental control. We have found that level of m*lip15* transcripts in maize increase with leaf aging (Berberich et al., 1999), indicating regulation not only by environmental factors but also by developmental cues. In order to examine whether or not this feature is generalized in other plant species, particularly in dicots, we analyzed two tobacco genes, *tbz17*, a counterpart of *lip19*, and *tbzF*, a second tobacco member of the *lip19* subfamily.

Materials and Methods

Plant materials and various treatments

Tobacco seeds (*N. tabacum* L. cv. Xanthi) were surface sterilized for 10 min in a 1% NaClO solution containing 0.1% of Tween-20, washed several times in sterile water and placed onto 0.5% agar containing half strength Murashige and Skoog (MS) medium in plastic containers (Sigma). Plants were grown to their 10th leaf stage in continuous white light (90 μE) at 25°C unless described otherwise. Plants were then transferred into a compound soil mixture of metromix/vermiculite/perlite (2:1:1) in a growth chamber (TOMY, Japan) with a 16h light/8h dark cycle and a temperature of 25°C. Stress and hormone application was with tobacco seedlings. For treatments directed to roots, the agar was carefully removed and the plants were transferred to liquid 1/2 strength MS medium and maintained for another 2 days under the same conditions before the start of any treatment. In the case of hormone treatments, abscisic acid (ABA, 20 μM), 6-benzylaminopurine (BA, 20 μM), (-) jasmonic acid (JA, 20 μM) or indole-3-acetic acid (IAA, 20 μM) were added to the 1/2 strength MS medium. For salt treatment, NaCl was added from a stock solution to the medium to give a 0.2 M final concentration. For ethylene treatment, plants were exposed to 10 μl/l ethylene gas in special glass containers. At each time point, 2nd and 3rd leaves from the tops of two plants were harvested, frozen in liquid nitrogen and stored at -80°C until use.

Isolation and characterization of a tbzF *cDNA clone*

A tobacco cDNA library from young flower buds was screened with the entire coding region of *tbz17* cDNA as a probe under a standard hybridization conditions as described earlier (Sambrook et al., 1989). Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) and assembled and analyzed with the SDC-GENETYX genetic information processing program (Lipman and Pearson, 1985; Software Development Co., Tokyo). Comparison with sequences in non-redundant databases was achieved with the BLAST program on network servers (Altschul et al., 1990).

Construction of GFP-fusion plasmids and fluorescence microscopy

Entire coding region fragments for *tbzF* and *tbz17*, sandwiched with *Xba*I and *Kpn*I sites, were subcloned into pGFP2 (provided by Drs. Chua and Spielhofer), resulting in pTBZF::GFP and pTBZ17::GFP, respectively. These plasmids encode fusion proteins with truncated TBZF and TBZ17 at the N-terminal portion and GFP at the C-terminal portion. Onion bulbs cut into 9 cm^2 were biolistically bombarded, as described previously (Hara et al., 2000), with gold particles (Bio-Rad) coated with the plasmids pGFP2, pTBZF::GFP or pTBZ17::GFP. After 6 h incubation at room temperature in complete darkness, the epidermal cell layers were viewed using a microscope (OLYMPUS PROVIS AX70) equipped with a fluorescence module.

Assay for transactivation activity in spinach leaves

GAL4-dependent transactivation assays in plants were performed according to the precedure described by Yamamoto and Deng (1998). The effector constructs encoding TBZF and TBZ17 derivatives fused to a GAL4 DNA binding domain were generated as follows; the corresponding regions of *tbzF* and *tbz17* cDNAs were amplified by PCR using appropriate primers, digested with *Bgl*II and *Xba*I, and the resulting fragments were subcloned into *Bgl*II and *Xba*I digested yy64 plasmids. All constructs were confirmed by direct sequencing. The reference plasmid pRTL2-GUS (Restrepo et al., 1990), which contains a GUS gene under a control of CaMV 35S dual promoter, was used to normalize for the efficiency of

bombardment. Spinach leaves were bombarded with gold particles (Bio-Rad) coated with reporter, reference and/or effector plasmids and incubated at 25°C for 12h under continuous light. Protein concentrations were measured using a Protein Assay kit (Bio-Rad) and luciferase activity with a PicaGene kit (Toyo Ink Co. Ltd). The GUS assay was carried out using an AURORA GUS assay kit (ICN Pharmaceuticals) and chemical luminescence was measured with a luminometer (Lumat LB9507; Berthold, Bad Wildbad, Germany).

Synthesis of TBZF and TBZ17 in E. coli

To prepare GST-TBZF and GST-TBZ17 fusion constructs, entire coding regions were amplified by PCR using *Pfu* polymerase (Stratagene). After *Bgl*II/*Sal*I digestions followed by gel purification, the fragments were cloned into the *Bam*HI-*Sal*I sites of pGEX-4X-1 (Amersham Pharmacia Biotech). The coding regions were amplified by PCR. Intactness of the junction sequences was confirmed by DNA sequencing. Recombinant TBZF and TBZ17 were prepared employing a GST purification module from Amersham Pharmacia Biotech according to the supplier's instruction. *E. coli* JM109 cells were transformed with the GST-TBZ constructs. To prepare bacterial extract, a single colony of transformed bacteria was inoculated in 2YT/Amp medium and grown overnight. The culture was diluted (1:100) into 250 ml of fresh media. Isopropyl-1-thio-D-galactopyranoside was added to the culture to a final concentration of 0.1 mM when A600 reached 0.7. Cells were harvested by centrifugation after further growth (1.5 h). The bacterial pellet was resuspended in 12.5 ml of phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and sonicated on a Astrason Ultrasonic processor (4 x 40-s burst at setting 5 at 80% duty cycle). The lysate was cleared of cell debris by centrifugation, and the supernatant was loaded onto a column packed with 0.125 ml (bed volume) of glutathione-Sepharose 4B resin. Wash and elution was performed as suggested by the supplier. Protein concentration was determined using the Bio-Rad protein assay kit. Production of GST-TBZ fusion protein was confirmed by Western blotting using GST antibody.

Electrophoretic mobility shift assay

The following oligonucleotides, A/G-hybrid (60 mer, 5'-GGCTTACGTGGGGCA-3'x4), C/G-hybrid (60 mer, 5'-TGGGGACGTGGCAGA-3' x4), mutated A/G-hybrid (60 mer, 5'-GGCTTTCGAGGGGCA-3' x4) and mutated C/G-hybrid (60 mer, 5'-TGGGGTCGAGGCA GA-3' x4), and their complementary oligonucleotides were synthesized, phosphorylated with [γ-^{32}P] ATP and T4 polynucleotide kinase, annealed and used as probes in electrophoretic mobility shift assay (EMSA). EMSA was performed as described (Kusano et al., 1995). All DNA-binding reactions were performed at 30°C in final volume of 15µl containing 4 mM Tris, pH 7.9, 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12 mM HEPES-NaOH (pH 7.9). Typically, BSA (0.3 mg/ml) and poly(dI)-(dC) (200 ng to 1 µg/reaction) were added to the binding reactions. The indicated proteins or extracts were incubated with 0.5-2 ng of probe (approximately 10,000-15,000 cpm) for 30 min at 30°C. Reactions were loaded onto a 4% polyacrylamide gels, which had been pre-run at constant voltage (100V) for more than 90 min, and electrophoresed at 30mA constant current under circulating buffer. Gels were dried and exposed to X-ray film for autoradiography.

Random binding site selection

The DNA sequence specificity of glutathione *S*-transferase (GST)-fused TBZF and TBZ17 proteins produced in *E. coli* JM109 was determined by a random binding selection assay, as described by Kosugi and Ohashi (2000) with a slight modification. A pool of 73-base oligonucleotides, BSS73, GTCTGTCTGAGGTGAGGGATCCTA(N)25ACAAGCTTG-TCTTCCGACATCGCG, where N is any nucleotide, was made double stranded by annealing with the primer 24-2 (5'-CGCGATGTCGGAAGACAAGCTTGT-3'), followed by extension using the Klenow fragment of DNA polymerase I. For the first round selection, 150 fmol of the double stranded BSS73 (R0 probe) was mixed with 1 µg of recombinant TBZ proteins in 15 µl of binding buffer (12 mM HEPES-NaOH, pH 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol containing BSA (0.3 mg/ml) and 2 µg of poly(dI)-(dC), and incubated for 30 min at 30°C. The protein-DNA complex was separated by polyacrylamide gel electrophoresis and the DNA was eluted from the gel and dissolved in 20 µl of TE. Amplification of the selected DNA was performed by PCR using 150 fmol each of

24-1 (5'-GTCTGTCTGAGGTGAGGGATCCTA-3') and 24-2 primers in a buffer (10 mM Tris, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$) containing 150 μM of each dNTP. The reaction was carried out for 25 cycles. Amplified DNA was separated by 8% polyacrylamide gel electrophoresis and the 73 bp-fragments were excised, eluted and used as probe DNA for the next round of selection. The selection cycle was repeated four more times with an increment of poly(dI)-(dC); second 3 μg, third and fourth 4 μg and fifth 5 μg. The DNA from the fifth round of selection was amplified by PCR and cloned into pGEM-T Easy vector (Promega) for sequencing.

Assay for plant transactivation activity

Plasmid N46 Luc+, in which CaMV 35S –46 minimal promoter was placed upstream of the *luc* reporter gene, was constructed as follows: two primers (5'-GGGAAGCTTAAGCTCGAG ATCTCGCAAGACCCTTCCTCTATATAAGGAAGTTC AT-3') and (5'-CGTACCATGGT CTAGACAGCGTGTCCTCTCCAAATGGAAATGAACTTCCTTATATAG-3') were annealed and filled-in with dNTPs and Klenow fragment, then digested with *Hin*dIII and *Nco*I. The resulting *Hin*dIII-*Nco*I fragment was subcloned into the respective sites of 221-Luc+ (Matsuo et al., 2001) to create N46 Luc+. Four reporter plasmids, A/G-W x4 Luc+ and C/G-W x4 Luc+ and their mutated derivatives, mA/G-M x4 Luc+ and mC/G-M x4 Luc+, were constructed as follows: The primer pairs of 60 bp described above were phosphorylated, annealed and cloned into *Hin*cII site of pBlueScript II SK-, and the respective recombinant plasmids were digested with *Hin*dIII and *Xho*I. The resulting *Hin*dIII-*Xho*I fragments were subcloned into the N46-Luc+ vector. GUS (β-glucuronidase) gene of the pBI221 (Clontech) was replaced with the *Xba*I-*Sac*I DNA fragments covering TBZF- and TBZ17-coding regions, resulting in pBI221-TBZF and pBI221-TBZ17. In case of pBI221-DEL GUS, the *Xba*I-*Sac*I fragment derived from the multicloning sites of the pBlueScript II SK- was inserted instead of GUS gene of the pBI221. The reference plasmid pRTL2-GUS (Restrepo et al., 1990), which contains a GUS gene under a control of CaMV 35S dual promoter, was used to normalize for the efficiency of bombardment.

Tobacco BY-2 cells (*N. tabacum* L. cv. Bright Yellow 2) were maintained as described

(Nagata et al., 1981). Five-day-old BY2 cells (2 ml) after subculture were spread on 3% agar plates and briefly allowed to dry to remove excess liquid. After bombardment, cells were harvested with 2 ml of liquid medium, transferred to a flask, and then incubated at 26°C with gentle agitation. Protein concentrations were measured using a Protein Assay kit (Bio-Rad) and luciferase activity with a PicaGene kit (Toyo Ink Co. Ltd). The GUS assay was carried out using an AURORA GUS assay kit (ICN Pharmaceuticals) and chemical luminescence was measured with a luminometer (Lumat LB9507; Berthold, Bad Wildbad, Germany).

Chlorophyll assays

Chlorophyll contents were measured as described in Chapter I.

RNA gel blot analysis

Extraction of total RNA and RNA blot analysis were carried out as described in Chapter I.

In situ RNA hybridization

In situ hybridization was performed using digoxigenin-labelled sense or antisense RNA produced from 3'-untranslated regions of *tbzF*- and *tbz17*-cDNAs. This plasmid, pTBZF and pTBZ17, were used as a template for synthesizing single-strand RNA. To generate sense and antisense-RNA probe, pTBZF and pTBZ17 were linearized with *Sal*I and an approximately 330-bp single-stranded RNA was synthesized by T7 or SP6 RNA polymerase using a DIG-RNA labeling kit (Boehringer Mannheim). Flower buds, young leves and senecing leves were fixed and paraffin embedded according to the method of Dixon et al. (1995). The samples were sectioned 10 μm thick, dewaxed, and rehydrated. The sections were incubated with proteinase K solution (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, and 20 μg mL^{-1} proteinase K) at 37°C for 30 min and then acetylated with 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0, for 5 min at room temperature. The sections were washed with 2 x SSC and dehydrated through an ethanol series. The sections were pre-hybridized in a hybridization solution (50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM $NaPO_4$, pH 8.0, 10 mM DTT, 5 mM EDTA, 1 x Denhardt's

solution, 500 μg mL^{-1} tRNA, 100 μg mL^{-1} salmon-sperm DNA, and 40 units mL^{-1} RNase inhibitor) at 48°C for 2 h, and hybridized with 1 μg mL^{-1} RNA probe at 48°C for 16 h. The sections were washed with 3 x SSC for 5 min at room temperature and incubated with NTE buffer (500 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5) containing 50 μg mL^{-1} RNase A at 37°C for 30 min. After RNase A treatment, sections were washed with NTE buffer three times for 5 min at room temperature and followed by washing with 0.2 x SSC at 60°C for 1 h.

For detection of hybridization signals, the sections were incubated in a color development solution (10% of polyvinyl alcohol [Mr 70,000-100,000], 100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM 5-bromo-4-chloro-3-indolylphosphate, and 0.2 mM nitroblue tetrazolium) at 30°C for 8 to 12 h. Color development was stopped with Tric-HCl (pH 8.0) and 1mM EDTA (pH 8.0). The slides were extensively washed with distilled water and dehydrated and mounted with nonaqueous mounting medium using standard protocols. Slides were photographed on a microscope (Nikon) using bright-field optics.

Results

Isolation of tbzF

During the course of investigation of transcript accumulation of *tbz17* with developmental growth, slightly larger transcripts were detected on northern hybridization using the *tbz17* cDNA as a probe. These larger transcripts were observed in a fraction of flowers (Fig. 14), suggesting the presence of another *tbz17* homolog in tobacco. For identification, a tobacco cDNA library derived from flowers was screened with the *tbz17* probe. All positive clones obtained originated from a gene distinct from *tbz17*, and designated as *tbzF* (*t*obacco *bz*ip protein in *F*lower). The nearly full-length *tbzF* cDNA was 1,099 bp and contained an ORF of 435 bp encoding a 144-amino acid protein (termed TBZF) with a relative molecular mass of 16.6 kDa (Fig. 15). Pairwise identities between TBZF and other *lip19* subfamily members were found to be 73% (TBZ17), 72% (rdLIP), 60% (Am910), 57%

(Am911), 52% (LIP19) and 46% (mLIP15) (Fig. 16A). Construction of a phylogenic tree indicated TBZF belongs to the *lip19* subfamily (Fig. 16B), characterized by small size, OBF1 being the largest at 151-amino acids, with 8-9 heptad leucine-repeats (Fig. 16A).

Nuclear localization

To examine the cellular localization of TBZF and TBZ17 proteins, cauliflower mosaic virus (CaMV) 35S promoter *tbzF*::*GFP* and CaMV 35S promoter *tbz17*::*GFP* genes were constructed and biolistically bombarded into onion epidermal cells. The fluorescent signals derived from these constructs were observed exclusively in nuclei (Fig. 17).

Transcriptional activation

Whether or not TBZF and TBZ17 have transactivation activity was tested employing a GAL4-dependent transactivation assay system (Fig. 18A) (Yamamoto and Deng, 1998). Expression of the reporter (*luc*) gene was enhanced about 8- and 10-fold, respectively (Fig. 18B and 18C). Three versions of TBZF truncates, even with deletion of 10 amino acids in the C-terminus, demonstrated diminished transactivation activity (Fig. 18B). In the case of TBZ17, with deletion of the amino-terminal half (TBZ17-Δ78), activation activity of about 90% was still retained. Further truncation to Δ99 or a 10 amino acid-truncation at the C-terminus reduced the activity to about 50% of the control (Fig. 18C). Almost identical results were obtained with a yeast system (data not shown). The results indicate that TBZF and TBZ17 proteins are transcriptional activators and their most carboxy-distal 10 amino acids are essential, but themselves not sufficient, for transactivation activity.

TBZF and TBZ17 bind to the G-box sequences

TBZF and TBZ17 were clearly related to the G-box element, a group that has been studied extensively in terms of DNA binding properties. I investigated whether TBZF and TBZ17 does so by electrophoretic mobility shift assays (EMSAs) using an *E. coli* crude extract containing TBZF and TBZ17 fused to GST. The EMSA using the G-box element from the *maf* probe (Fig. 19A). The retard bands were producted when *maf* probe was incubated

with protein extracts drived from *E. coli* cells expressing the TBZF and TBZ17 recombinat proteins (Fig. 19B). Binding specificity was demonstrated by competition titrations up to 40-fold concentration with the same unlabelled oligonucleotides used for binding (Fig. 19B).

DNA binding specificity and transcriptional activation

To determine the target DNA sequences of TBZF and TBZ17 homodimers in vitro, random binding site selection assay was performed (Kosugi and Ohashi, 2000). With repeated selection assay, the target sequences were enriched (Figs 20A and 20B). The respective enriched fragments after selection of the fifth round were subcloned into pGEM-T Easy vector and determined by DNA sequencing. Preferred DNA sequences of TBZF and TBZ17 were classified into 3 types each (Figs 20C and 20D).

Both preferred sequences containing an A/G hybrid box (5'-**T**ACGT**G**-3') and a G/C hybrid box (5'-**G**ACGT**G**-3'). Using one of each of the enriched A/G- and C/G- hybrid box sequences, electrophoretic mobility shift assays were performed. TBZF and TBZ17 bound to the A/G- and C/G-hybrid box sequences, but not to their mutated sequences (Fig. 21).

When these A/G- and C/G-hybrid box sequences were fused to the CaMV –46 minimal promoter driving the *luc* (luciferase) reporter gene, and co-bombarded with TBZF- and TBZ17-producing effector plasmids into tobacco BY2 cells, expression of the reporter genes was enhanced about 14- to 16-fold (Fig. 22). These results indicate that TBZF and TBZ17 proteins efficiently bind to DNA fragments spanning A-box/G-box and C-box/G-box hybrid motifs.

Stress and hormonal responses

Rice *lip19* was first identified as a low-temperature-responsive gene (Aguan et al., 1993). To address the responses of *tbz* genes to abiotic stress, northern hybridization analysis was performed (Fig. 23). The *tbzF* transcripts were profoundly upregulated by 4°C exposure for 6h but heat shock had no effect. Drying for 1h upregulated *tbzF* expression but high salt did not induce transcripts. In contrast, *tbz17* was mildly upregulated by low temperature, whereas other treatments were without obvious influence (Fig. 23A). Investigation of effects

of phytohormones demonstrated *tbzF* and *tbz17* transcripts to be upregulated by ethylene, IAA and JA, but not by BA (Fig. 23B).

Transcript accumulation in senescing leaves and flowers

Developmental change in *tbzF* and *tbz17* transcript levels was analyzed in matured tobacco plants bearing flower buds. In flowers, the *tbzF* mRNA expression was high, whereas that of *tbz17* was low. In roots, *tbzF* transcripts were detected at low levels, whereas *tbz17* transcripts were absent (Fig. 24). In leaves, the levels of *tbzF* and *tbz17* transcripts were inversely proportional to the chlorophyll content (Fig. 24). The results indicate both *tbzF* and *tbz17* to be novel senescence-associated genes.

Accumulation of tbzF transcripts in floral organs

Monitoring of *tbzF* transcripts during flower development by northern analysis revealed a constant presence until the stage 6 of full expansion of petals, but just after petal shrinkage (stage 7) levels rapidly decreased (Fig. 25A). Transcripts of *tbzF* were ubiquitously detected in all flower organs except pistils (Fig. 25B). *In situ* RNA hybridization clearly showed that the temporal and spatial accumulation of *tbzF* transcripts were mainly in sepals, petals, stamens, ovaries and pistils (Fig. 25C). Some signals were also detected in the vascular tissues of the sepals and the stems of the young inflorescence.

Predominance of transcripts in guard cells

To test spatial expression of *tbzF* and *tbz17* in senescing leaves, *in situ* RNA hybridization was performed. With antisense probes, very intense signals were observed for both *tbzF* and *tbz17* in guard cells of senescing leaves, whereas only background levels of signals were detected in mesophyll cells. Their transcripts were detected in both abaxial and adaxial stomatal guard cells (Fig. 26, c-d and i-j), and also within the epidermal cell layer of mature leaves, although signals were much weaker. Consistent with the northern results (Fig. 24), very low signals were detected in young leaves (Fig. 26, a-b and g-h). We further confirmed the guard cell-specific expression of *tbzF* and *tbz17* using horizontal cross-sections

of mature leaves (Fig. 26, e-f and k-l).

Discussion

Most members of the *lip19* subfamily are characterized by low-temperature induction and transcripts of *tbzF* and *tbz17* also accumulate at low temperature. In addition to this stress-response, expression was found to be positively controlled with leaf development, abundance inversely correlating with the total chlorophyll content in leaves (Fig. 24). It should be emphasized that these bZIP-genes are senescence-associated (Smart, 1994) and my study revealed transcripts of *tbzF* and *tbz17* to be predominantly expressed in guard cells of senescing leaves. To our knowledge this is the first report of such preferential expression of bZIP-type transcription factor-encoding genes. Arabidopsis *TGA6,* encoding a bZIP protein of the TGA family, is expressed in aging cotyledons, vascular tissue and trichomes of senescing rosette leaves, in lateral roots and mature pollen grains but not in guard cells (Xiang et al., 1997). In the case of *tbzF*, predominant expression in vascular tissues and trichomes in petals and sepals of flowers was also observed.

Martínez-García et al (1998) pointed out that *lip19* subfamily members have a conserved upstream open reading frame (uORF) in their 5' leader sequences, and suggested a common mode of post-transcriptional control. This feature was also observed in both *tbzF* and *tbz17* cDNAs; *tbzF* and *tbz17* contain uORFs of 25- and 28-amino acids, respectively, and the uORFs show high identity to those of other *lip19* subfamily members.

In this Chapter, I suggested that TBZF, the product of a novel bZIP-gene from tobacco, binds to hybrid sequences of A-box/G-box and C-box/G-box motifs and can function as a transcriptional activator. TBZ17, the product of the previously isolated-tobacco member of the *lip19* subfamily, shows 73% identity to TBZF and has a similar DNA binding specificity and potential for transactivation. *A. majus* bZIP proteins, 910 and 911, prefer DNA sequences containing a hybrid C-box/G-box motif (Martínez-García et al., 1998) and the products of the *lip19* subfamily may bind primarily to hybrid sequences of C-box/G-box and/or A-box/G-box

motifs when they take a homodimer form. As a worldwide-effort, DNA sequences of the Arabidopsis genome have been completely determined. A search for TBZF- and TBZ17-recognizing sequences on Arabidopsis chromosomes II and IV reveals that there are approximately 700 sites on each, under the condition of monitoring regions within 1 kb 5' upstream of each gene. It is, therefore, difficult to predict target genes for TBZ proteins only through genomic sequence information. However, another cue could be obtained from their specific expression in guard cells.

Stomatal guard cells are highly specialized and differentiated plant cells that play a critical role in metabolism by modulating gas exchange in photosynthetic tissues. They respond to various endogenous and exogenous signals such as light, humidity, CO_2 concentration and hormones (Assmann, 1993) and a sophisticated gene network is likely to be essential to control the metabolism of the stomatal complex, although my knowledge is as yet quite limited (Müller-Röber et al., 1998). With the aim of obtaining insights into this system, construction of a guard cell-specific cDNA library and massive DNA sequencing of the expressed clones have been started (Kopka et al., 1997; Kwak et al., 1997). Genes expressed in guard cells can be classified into at least 4 groups; the first encodes signal transduction components, such as AAPK (ABA-activated serine-threonine protein kinase, Li et al., 2000) and RHA1 (Ypt/Rab type-small G protein, Terryn et al., 1993). The second encodes channel components like KAT1 and KST1 (inward-rectifying K+ channel, Müller-Röber et al., 1995), PMA4 (H+-ATPase, Moriau et al., 1993) and HAAP (aquaporin, Sarda et al., 1997). The third group comprises structural protein-genes like StGCPRP (proline-rich protein, Menke et al., 2000) and GRP1 (glycine-rich protein, Smart et al., 2000), while the last includes defense-related genes such as *kin1* and *cor6.6* (Kurkela and Frank, 1990; Gilmour et al., 1992), the ABA-responsive *CdeT6-19* (Taylor et al., 1995), an acidic chitinase-gene (Samac and Shah, 1991), a lipid transfer protein-gene (Smart et al., 2000) and a kind of dehydrin-gene, *tas14* (Parra et al., 1996). Among the four, the last group seems to provide the most likely candidates as targets of TBZF or TBZ17 since *kin1* and *cor6.6* are also responsive to low-temperature (Kurkela et al,, 1990; Gilmour et al., 1992). Furthermore, transgenic plants with *kin1* and *cor6.6* promoter-GUS constructs demonstrate strong GUS activity in guard cells,

pollen and trichomes which increased with leaf age (Wang and Cutler, 1995). The expression pattern of *tbzF* and *tbz17* described here is strikingly similar to those of *A. thaliana kin1* and *cor6.6* genes. While induction of the latter genes by cold and drought has been explained by an interaction between a DRE/C-repeat element and DREBP/CBF1 protein (Stockinger et al., 1997; Liu et al., 1998), cooperation between these regulatory factors and TBZ proteins in regulation of *kin1* and *cor6.6* gene expression is possible.

What could be the function of *tbzF/tbz17* in guard cells of senescing leaves? One of the distinct features of guard cells is that they are the only cell type in the leaf epidermal cell layer which contains chloroplasts and are therefore photosynthetically active. It could be possible that *tbzF/tbz17* expression is associated with the loss of photosynthetic activity in these specialized cells during leaf senescence. On the other hand stomata of senescing leaves remain operable well after the mesophyll cells of the leaf have turned yellow (Willmer and Fricker, 1996). It is also probable that TBZF/TBZ17 may play a role in senescence retardation in guard cells enabling these cells to respond to environmental and endogenous stimuli although the rest of the leaf tissue is re-programmed for degradation. The products of *tbzF* and *tbz17* may activate unidentified genes which function to retain cellular activity in senescing- and cold-stressed-guard cells and/or vascular tissues of flowers, although it remains to be clarified whether their expression is upregulated in guard cells upon exposure to low temperature.

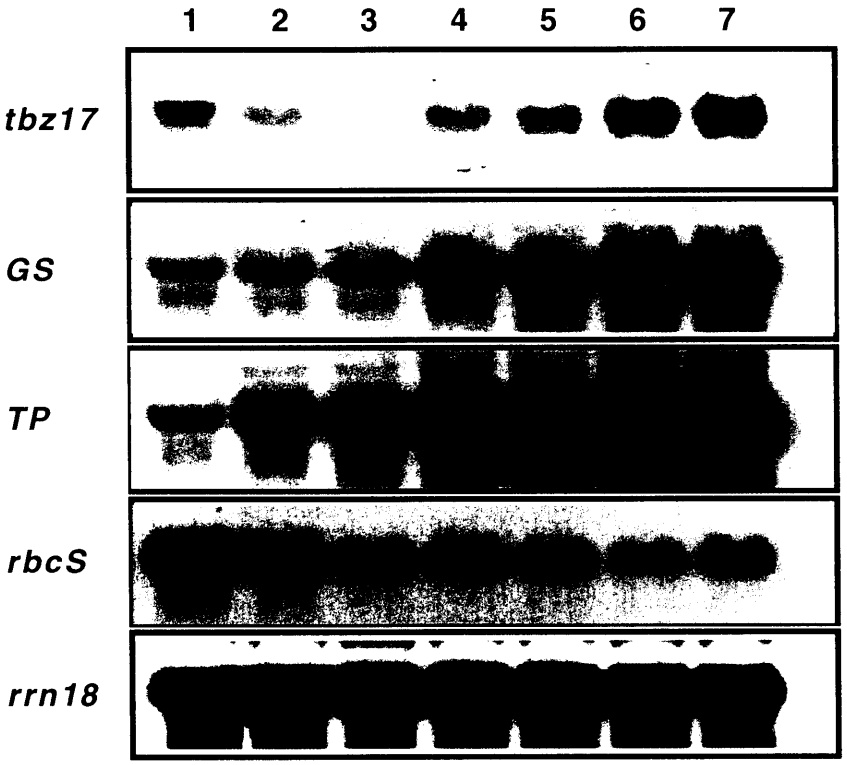


Fig. 14 Expression pattern of *tbz17* in different leaves, revealed by RNA gel blotting. Vegitative tobacco leaves were havested and numbered sequentially following thier progressive emergence. Total RNAs were also extracted from the same tissues and the abundance of *tbz17* transcript was estimated by northern hybridization using the respective ORF regions. The same filter was subsequently hybridized with the tobacco *actin* cDNA (*actin*, Thangavelu et al., 1993) to ensure equal sample loading.

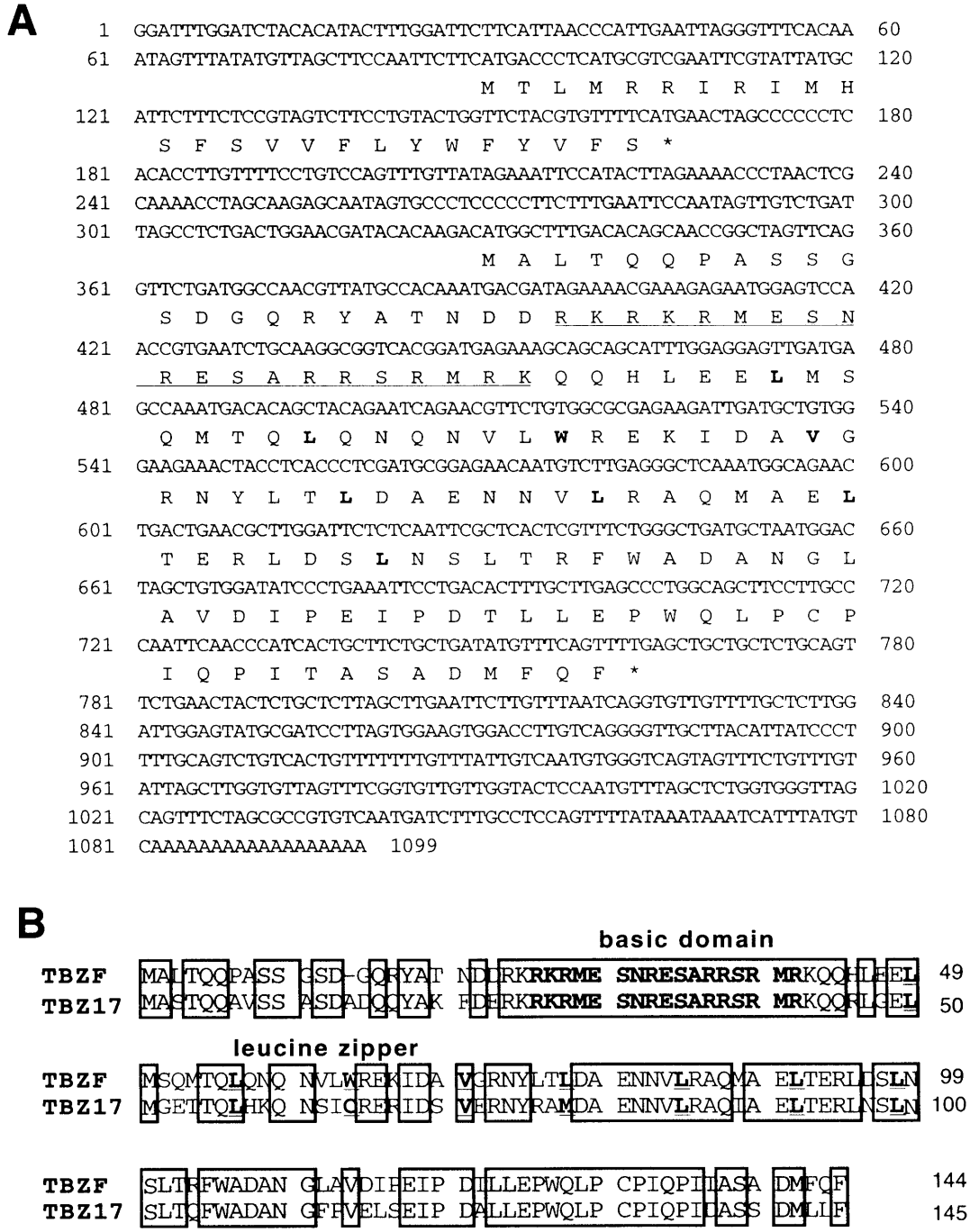


Fig. 15 Sequence analysis of *tbzF*. (**A**) DNA sequence and deduced amino acid sequence of *tbzF*.Numbers on the right correspond to the nucleotide positions. The predicted amino acid sequences of the main *bZIP* ORFs and the ORFs of the leader sequences are shown. The basic domain is underlined and the haptad repeats of hydrophobic residues in the putative leucine zipper structure are bold. (**A**) Alignment of the amino acid sequences for TBZF and TBZ17 proteins. The basic domain is bold and the haptad repeats of hydrophobic residues in the putative leucine zipper structure are underlined.

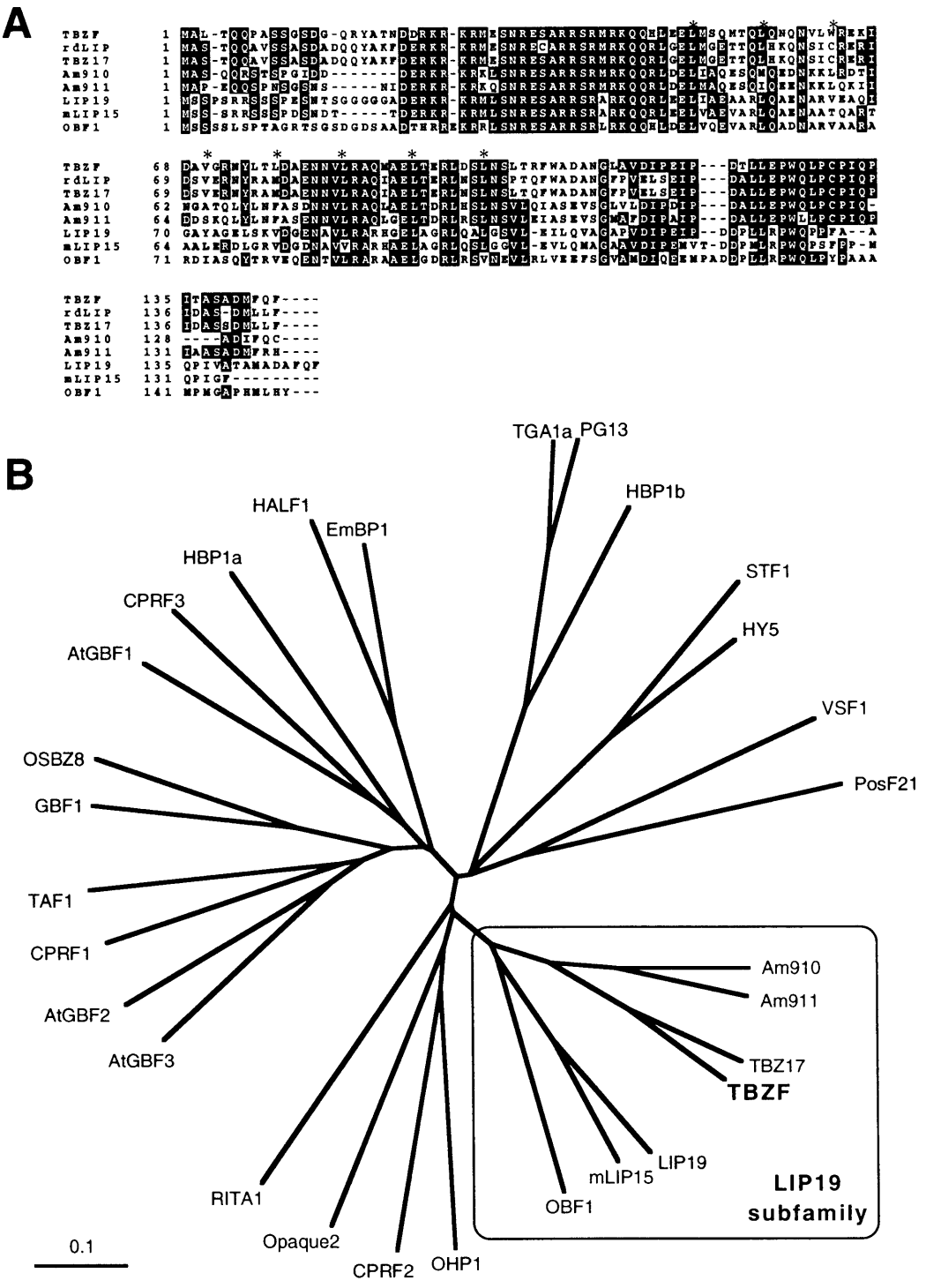


Fig. 16 (**A**) Amino acid sequence alignment of TBZF and LIP19 subfamily proteins. Identical residues are highlighted in black and similar residue in grey. Dashes were introduced to maximize the alignment. A basic region and the positions of heptad leucine residues are indicated by hooked line and asterisks, respectively. TBZF, rdLIP (AB005187, Ito et al., 1999), TBZ17 (D63951, Kusano et al., 1998), Am910 (T17108) and Am911 (T17110) (Martínez-García et al., 1998), LIP19 (X57325, Aguan et al., 1993), mLIP15 (D26563, Kusano et al., 1995) and OBF1 (X62745, Singh et al., 1990). (**B**) A phylogenic tree of plant bZIP proteins. Trees were built using Clustal X program (Thompson et al., 1997) and visualized by a TreeView program (Page, 1996). Accession numbers are: AtGBF1-3 (X63894-X63896), CPRF1-3 (X58575-X58577), EmBP1 (P25032), GBF1 (U10270), HALF1 (D64051), HBP1a (X56781) and HBP1b (X56782), HY5 (AB005456), OHP1 (L00623), Opaque 2 (X15544, M29411), OSBZ8 (U42208), PG13 (M62855), PosF21 (X61031), RITA1 (L34551), STF1 (L28003), TAF1 (X60363), TGA1a (S05452), TGA1b (S05453) and VSF1 (X73635). LIP19 subfamily was squared and TBZF was highlighted by bold-letter. The bar corresponds to 0.1 Jukes-Cantor substitutions per nucleotide.

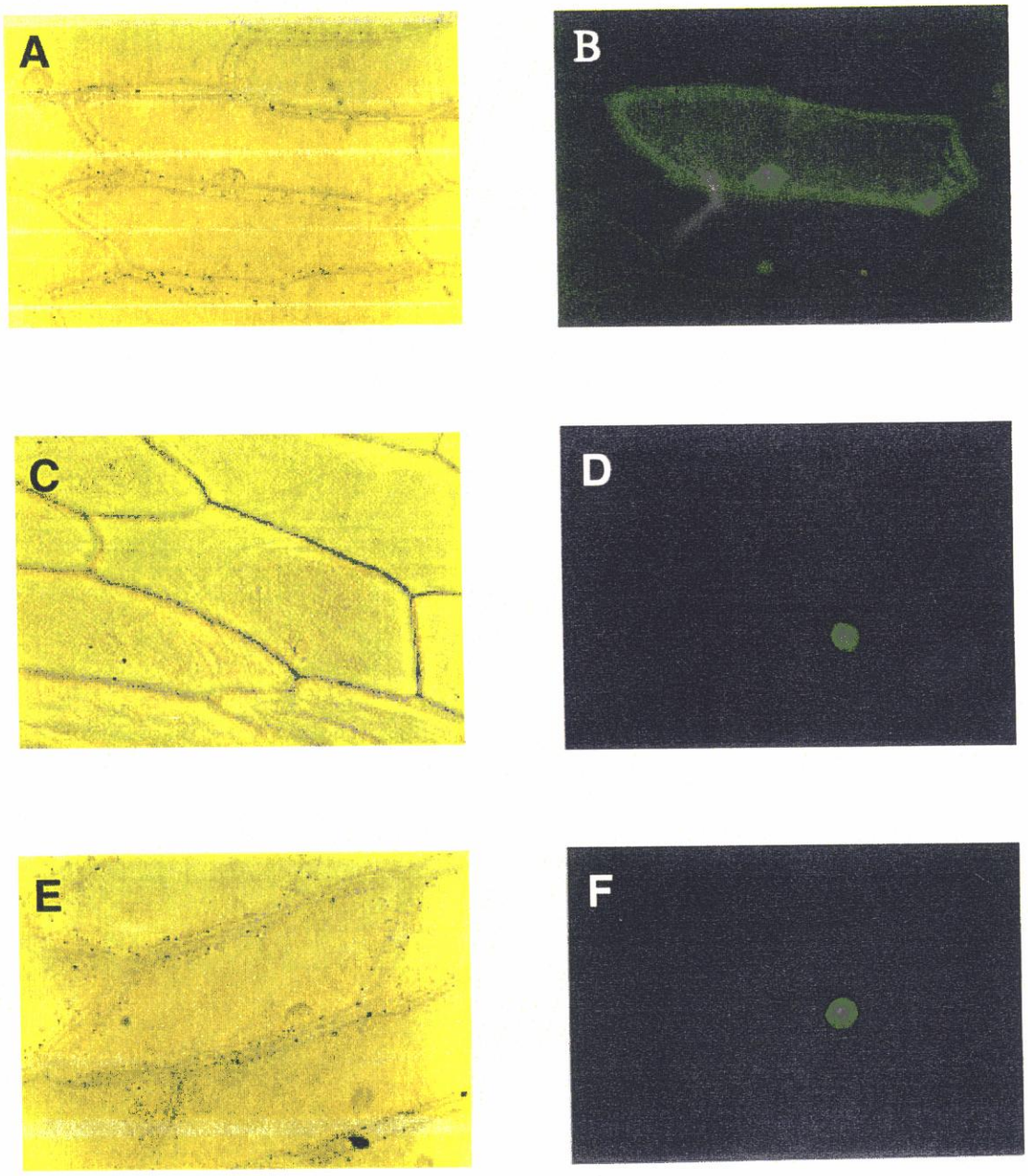


Fig. 17 Nuclear localization of TBZF and TBZ17 in onion epidermal cells. Onion bulbs were bombarded with gold particles coated with (**A, B**) pGFP2, (**C, D**) pTBZF::GFP, and (**E, F**) pTBZ17::GFP plasmids. The proteins were transiently expressed and individual cells are observed by epifluorescence (**B, D, F**) and corresponding differential interference contrast imaging (**A, C, E**).

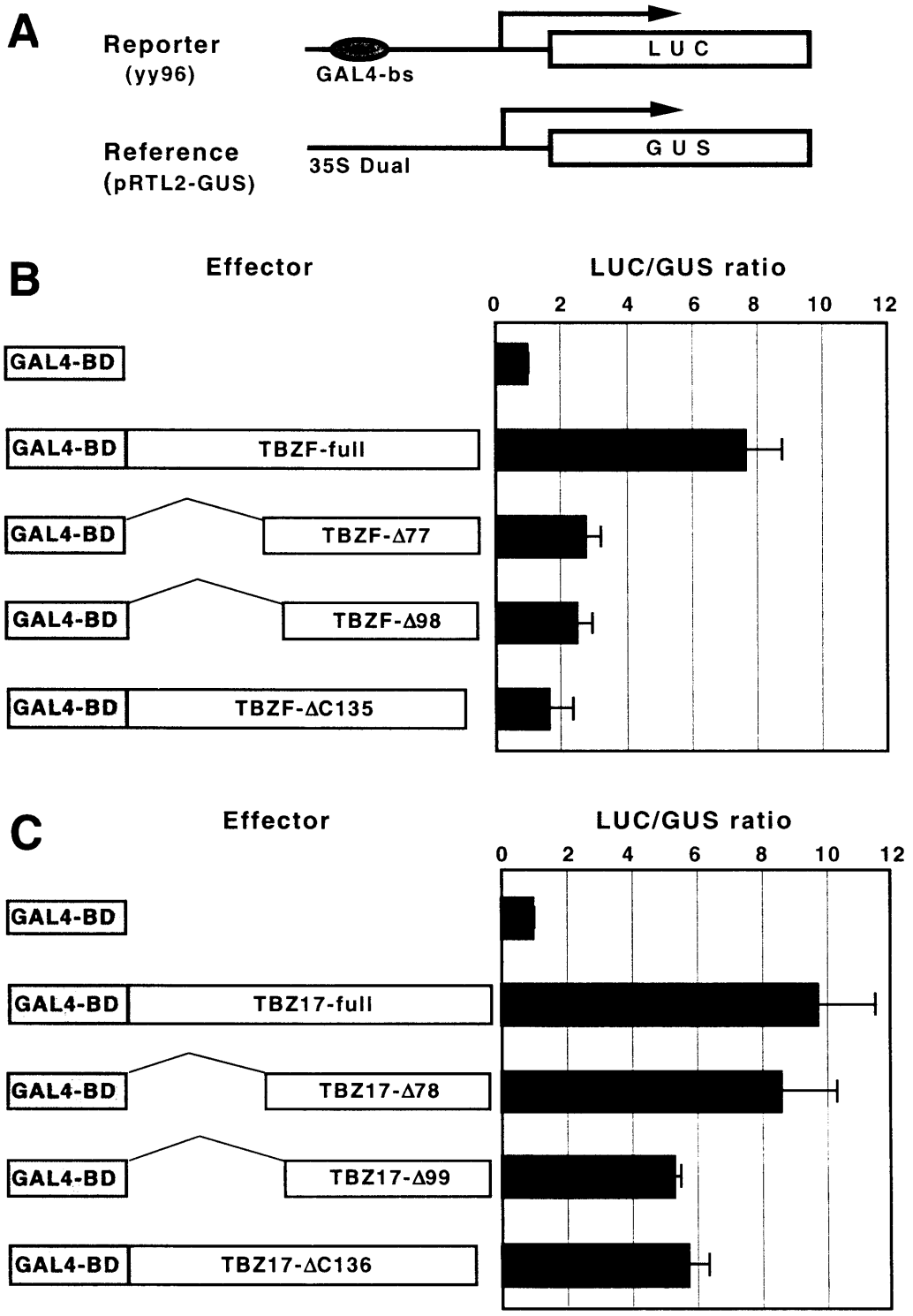


Fig. 18 TBZF and TBZ17 act as transcriptional activators in spinach leaves. (**A**) Schematic illustration of a reporter plasmid yy96 (Yamamoto and Deng, 1998), in which the luciferase gene (LUC) is placed downstream of the GAL4-binding site (GAL4-bs) and the reference plasmid pRTL2-GUS (Restrepo et al., 1990) which has a dual 35S promoter-GUS structure. (**B**) Transactivation activity of TBZF and its derivatives. (**C**) Transactivation activity of TBZ17 and its derivatives. (**B**) and (**C**) Effector, reporter and reference plasmids were cotransformed into spinach leaves by particle bombardment. After 24 h incubation at 25°C, LUC and GUS activities were determined. The LUC/GUS ratio obtained with pGAL4-BD (binding domain) was set as 1.0 to allow relative values to be obtained. The presented data are averages of three independent measurements in duplicate with SD error bars.

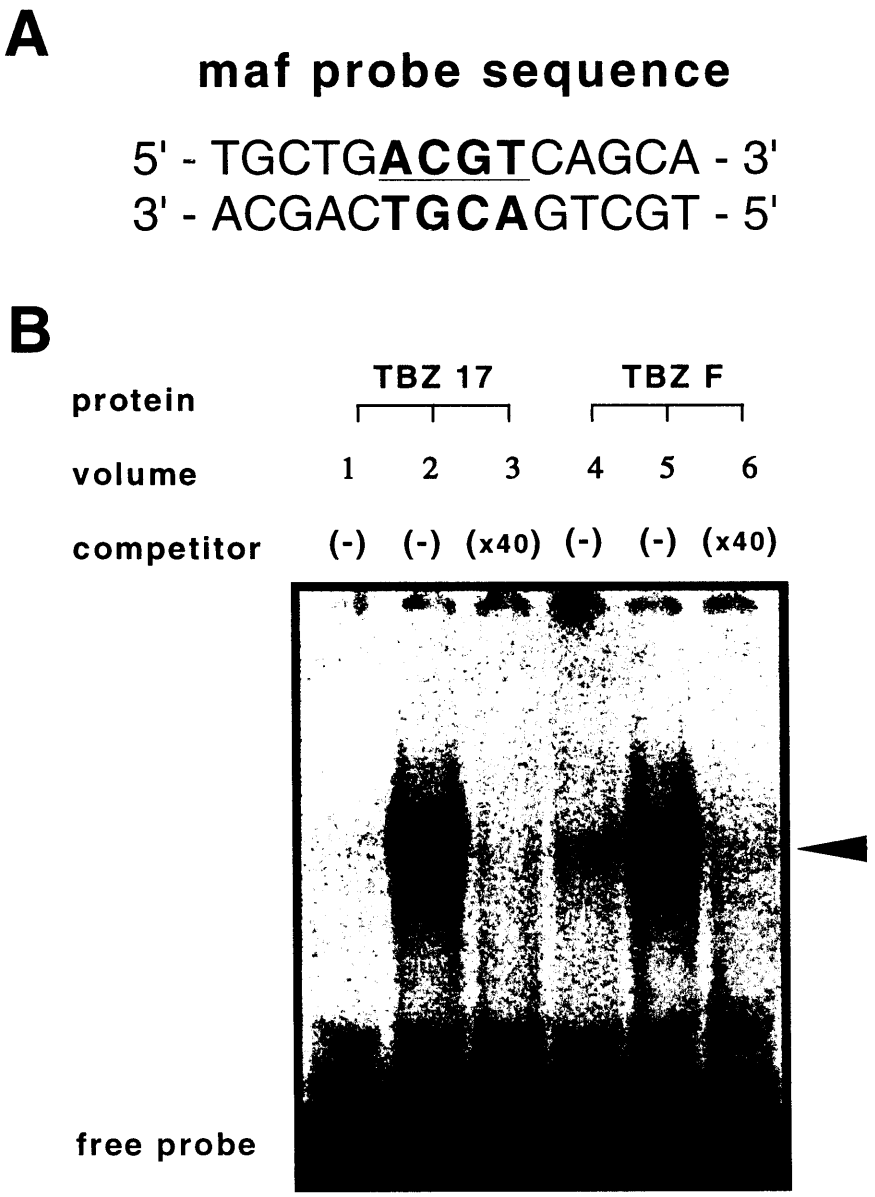
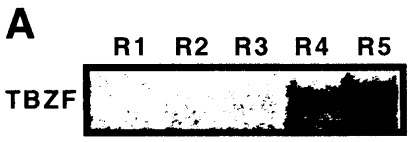


Fig. 19 Electrophoretic mobilitiy shift assays with synthetic oligonucleotides. (**A**) Nucleotide sequences of DNA probe with G-box. The ACGT motif is underlined. (**B**) 2μl of TBZF and TBZ17 (lanes 1, 4), and 8 μl of TBZF and TBZ17 (lanes 2, 3, 5, 6). Shifted complex of TBZF and TBZ17 proteins is indicated by arrows. (-), omission from the competitor; (x40), addition to the 40x concentration of competitor.



C **TBZF**

Type 1

TACGTG

GGATCCTACGTGGAACTTTC	(F3)
GGATCCTACGTGGTATGTTC	(F4)
GGATCCTAGGTGGGAGTGTG	(F5)
GGATCCTACGTGGTGTGTTT	(F6)
GGATCCTACGTGGCGTGATT	(F11)
GGATCCTACGTGTACTGTGT	(F14)
GGATCCTACGTGGCGGTGTT	(F17)
GGATCCTACGTGGTGTTACG	(F23)
GGATCCTACATCTGTCTTAT	(F24)
AAGTGGTACGTGCAGGCATA	(F31)
GAGGAATACGTCTGTAGTCT	(F39)
CAAGCGTACGTGCCTCCATT	(F43)
ATGTCGTACGTGTCATGCCA	(F45)
TCACAATACGTGCACACTCC	(F46)
GGATCCTACGTGGATACTTA	(F47)
GGATCCTACGTGCAACGCCA	(F48)
GGATCCTACGTGGGAGGAAG	(F53)

Type 2

SACGTG

GGATCCACGTGGTGGTGGG	(F13)
GGATCCA-GTGAGCAACTC	(F30)
GGTTGGACGTGGAAGCTT	(F34)
AATGGCACGTGCGGGATTA	(F42)
GGTTGGACGTGACAAGCTT	(F44)
TGACGGACGTGGACAAGCT	(F49)
CGATGGACGTGCAGACAAG	(F56)
CTTGTGACGTGGGTCGATG	(F60)
GGATCCACGTGGTCGTTCT	(F62)
ATTCTGACGTGGATCC	(F64)

Type 3

TACGTT

GATCGGATGTTAATTATGT	(F1)
CTACGCA-GTTAANTGCAC	(F2)
GATCCTA-GTCGTGGAGGC	(F58)
GGGTGTACGTTAAGACAAG	(F15)
ATAGCTACGTTGGATCGCA	(F25)
ACAAGTAAGTTTACAAACA	(F40)
ATCCATACGTTCTGTCTTT	(F61)

D **TBZ17**

Type 1

TACGTG

AGCTCTTACCTGAGTTACAAGCTT	(17.2a)
GGATCCTACGTGTGCGGTTGTGTT	(17.3)
GTGGCTTACGTGGGGCACAAGCTT	(17.4)
GGATCCTATGTGGTCACTTGGCCC	(17.2)
GGATCCTACGTGTAGAGTTGTCGC	(17.9)
GGATCCTATGTCAGTCGCTTCTGA	(17.6)
GGATCCTATGTGGAAGGGGATCGT	(17.10)
GGATCCTACGTGCAAACGGCTGGG	(17.11)
CACCGCTACGTCCGCGCAGCACAC	(17.23)
GGATCCTACGTGCCGGTGGTTGCT	(17.27)
AGCTTGTACGTCTGAGAACAAGCC	(17.37)
TGTCTCTACGTCATGCCATGTTGC	(17.38)
TACGTCACGTGCAAGCTGTTTTGA	(17.44)
TGTATGTACGTGTGCTGATGTGGC	(17.45)
GGATCCTAC-TGGTATGCTCTCGT	(17.4)
GGATCCTA-GTGTACTAGCAAGCA	(17.40)

Type 2

GACGTG

TGTGGGGACATGGACAAGCTT	(17.5)
TTTGGGGACGTGGCTGCATGG	(17.8)
GTGTGGGACGTGGTGGGGACA	(17.12)
AGCAATGACGTGGCAGGCTAG	(17.15)
GTTGGTGACGTGGGAGCGTGT	(17.26)
TCCTAGGACGTGGTCGGATTC	(17.32)
TCCGATGACGTGAGACAAGCT	(17.34)
GTTGGTGACGTGGGAGCGTGT	(17.36)
TAGGTAGAC-TGTGGCGTGTT	(17.5)
ACACACGAC-TGCCACCATAG	(17.25)
TTGTTACACGTGACACACGAC	(17.35)

Type 3

NACGTT

CTAGCGGAG-TTGTCAGAATCG	(17.1a)
CTCGCTGACGTTATTTTGGCTG	(17.1)
TGTGGCCACGTTCACTCGTTCC	(17.9a)
TCAGTGCACGTTGATGCGGATT	(17.28)
AAGGGCGACGTTGTCTATGCAG	(17.46)
AACCGCTA-GTTGCAGCCTAG	(17.41)
CTTCTCTACGTTACGGGCACAA	(17.42)

Fig. 20 Enrichment of target sequences of TBZF and TBZ17 by random binding site selection assays. GST-fused TBZF and TBZ17 produced in *E. coli* and purified by glutathione-Sepharose 4B were used for the assays. Enriched fragments (100 fmoles each) with every round (R1-R5) were used as probes for gel mobility shift assays and the parts of the gels containing the binding complexes of TBZF (**A**) and TBZ17 (**B**) are shown. Enriched sequences with TBZF (C) and TBZ17 (**D**) were classified into three types, respectively.

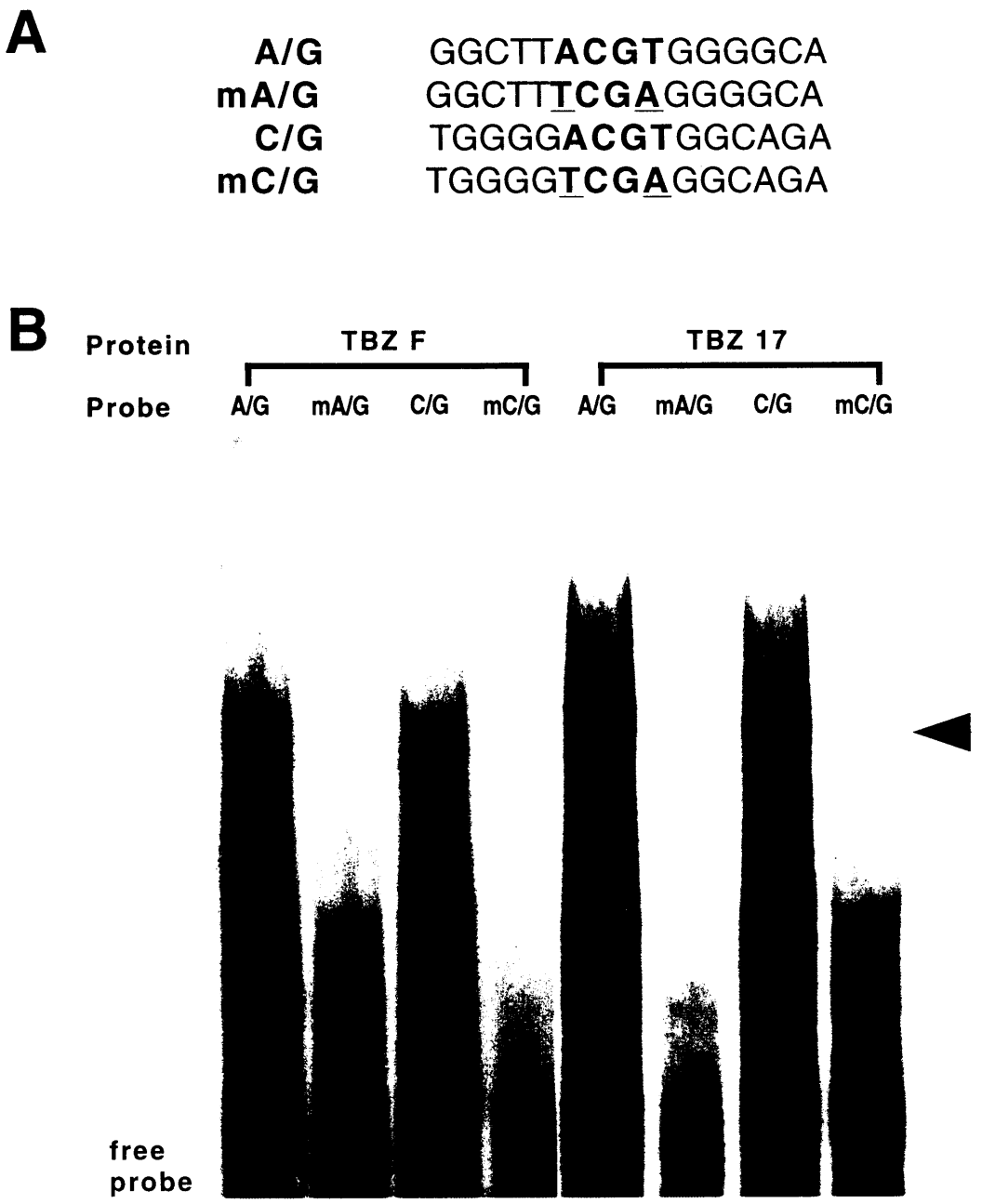


Fig. 21 TBZF and TBZ17 preferentially bind to DNA sequences containing A/G- and C/G-hybrid motifs. (**A**) A/G- and C/G-hybrid motifs and their mutated motif sequences (mA/G and mC/G) used for EMSA. The mutated nucleotides were underlined. The sequences used were 60 bp, tandemly repeated four times. (**B**) EMSA of the recombinant TBZF and TBZ17 proteins. The positions of the DNA-protein complexes were indicated by arrowhead.

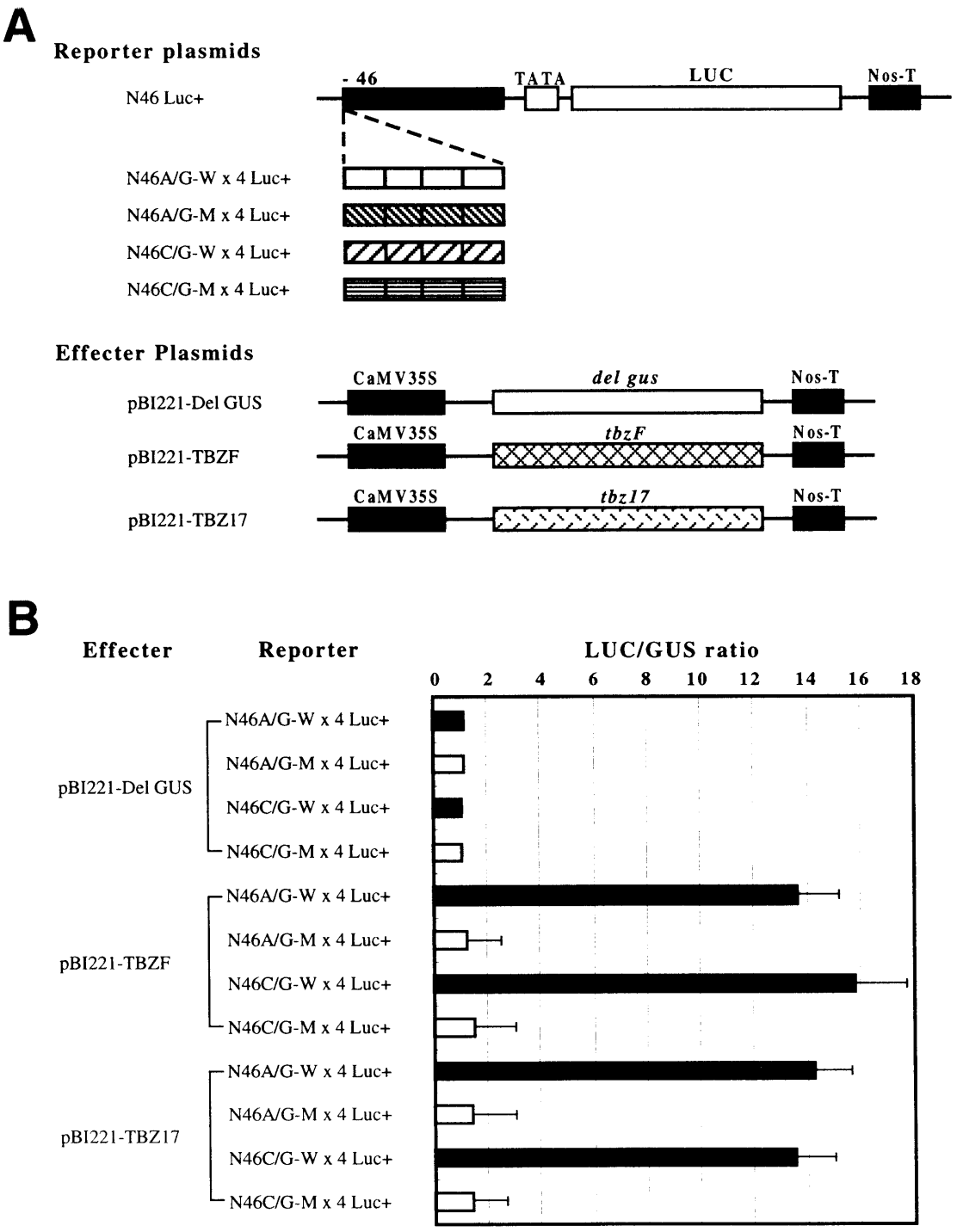


Fig. 22 TBZF and TBZ17 act as transcriptional activators in tobacco BY2 cells. (**A**) Schematic draw of a basal reporter plasmid N46 Luc+ and its four different derivatives with A/G-hybrid and C/G-hybrid *cis* motifs and their respective mutated motifs shown in Fig. 3. In N46 Luc+, the luciferase gene (*luc*) is placed downstream of the CaMV –46 minimal promoter (-46), (**B**) Transactivation activity assays of TBZF and TBZ17. Effector, reporter and reference plasmids were co-bombarded into BY2 cells by particle delivery system (PDS-1000 He, Bio-rad). After 18 h incubation at 26°C, LUC and GUS activities were determined. LUC/GUS ratio obtained by a combination of pBI221-Del GUS and N46A/G-W x4 Luc+ was set as 1.0 and the relative LUC/GUS values obtained from three independent experiments in duplicate assays were calculated with standard errors.

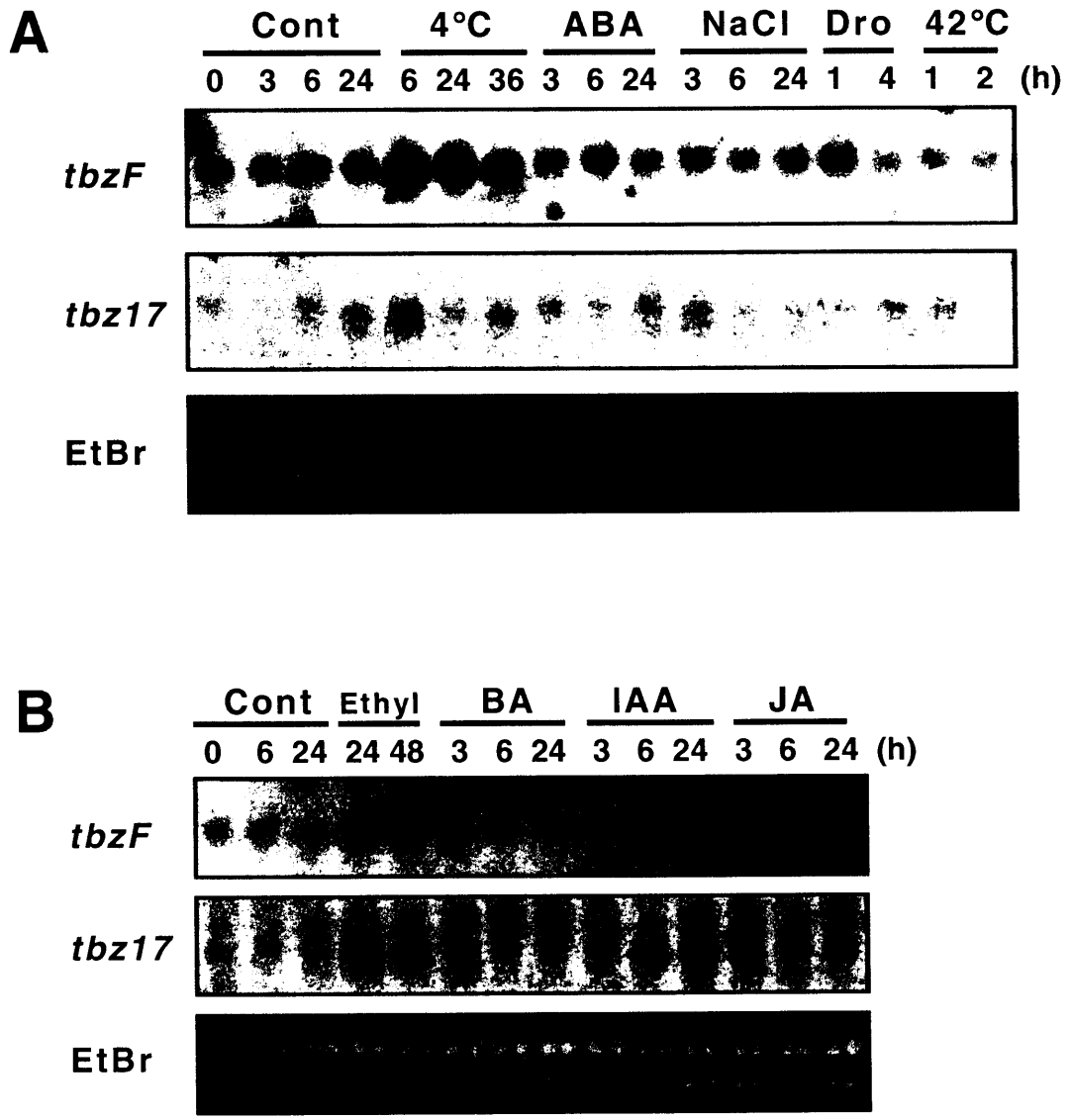


Fig. 23 Response of *tbzF* and *tbz17* genes in tobacco plants subjected to several abiotic stresses- and hormonal treatments. Tobacco seedlings were subjected to abiotic stresses and hormone treatment as described in Materials and Methods. (**A**) control (Cont), 4°C, abscisic acid (ABA), 0.2 M NaCl-treated (NaCl), air-dried (Dro) and heat shocked (42°C). (**B**) ethylene-treated (Ethyl), benzyladenine (BA), indole acetic acid (IAA) and jasmonic acid (JA).

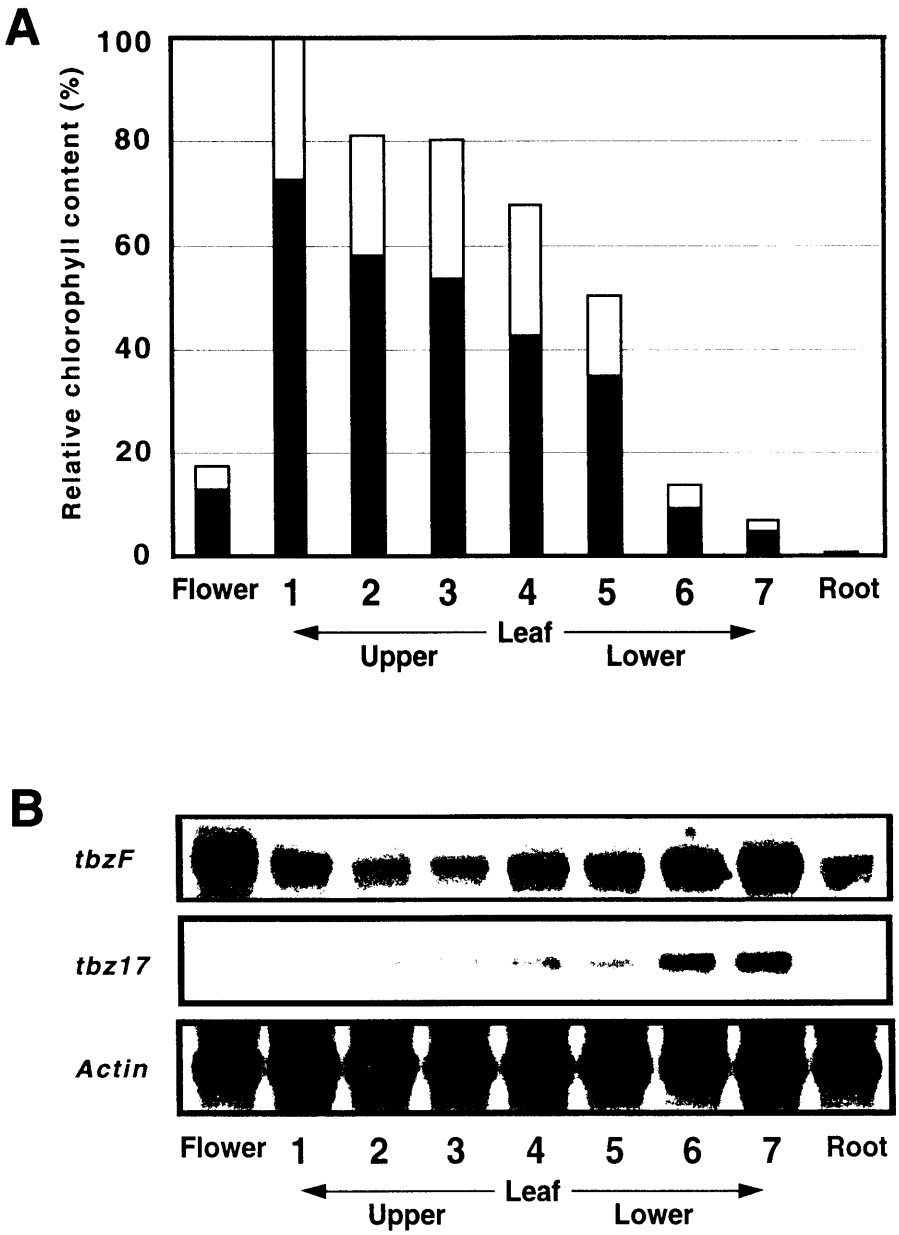


Fig. 24 Tissue specific expression of *tbzF* and *tbz17* in a mature tobacco plant. Tobacco plant was grown in a greenhouse. Various tissues including seven different positions of leaves, young flower buds and roots of a tobacco plant were harvested around noon of sunny day and kept at -80°C until use. (**A**) Chlorophyll a (filled bar) and b (open bar) contents of various tissues were measured as described in Materials and Methods. (**B**) Total RNAs were also extracted from the same tissues and the abundance of *tbzF* and *tbz17* transcripts were estimated by northern hybridization using the respective 3'-UTR regions. The same filter was subsequently hybridized with the tobacco *actin* cDNA (*actin*, Thangavelu et al., 1993) to ensure equal sample loading.

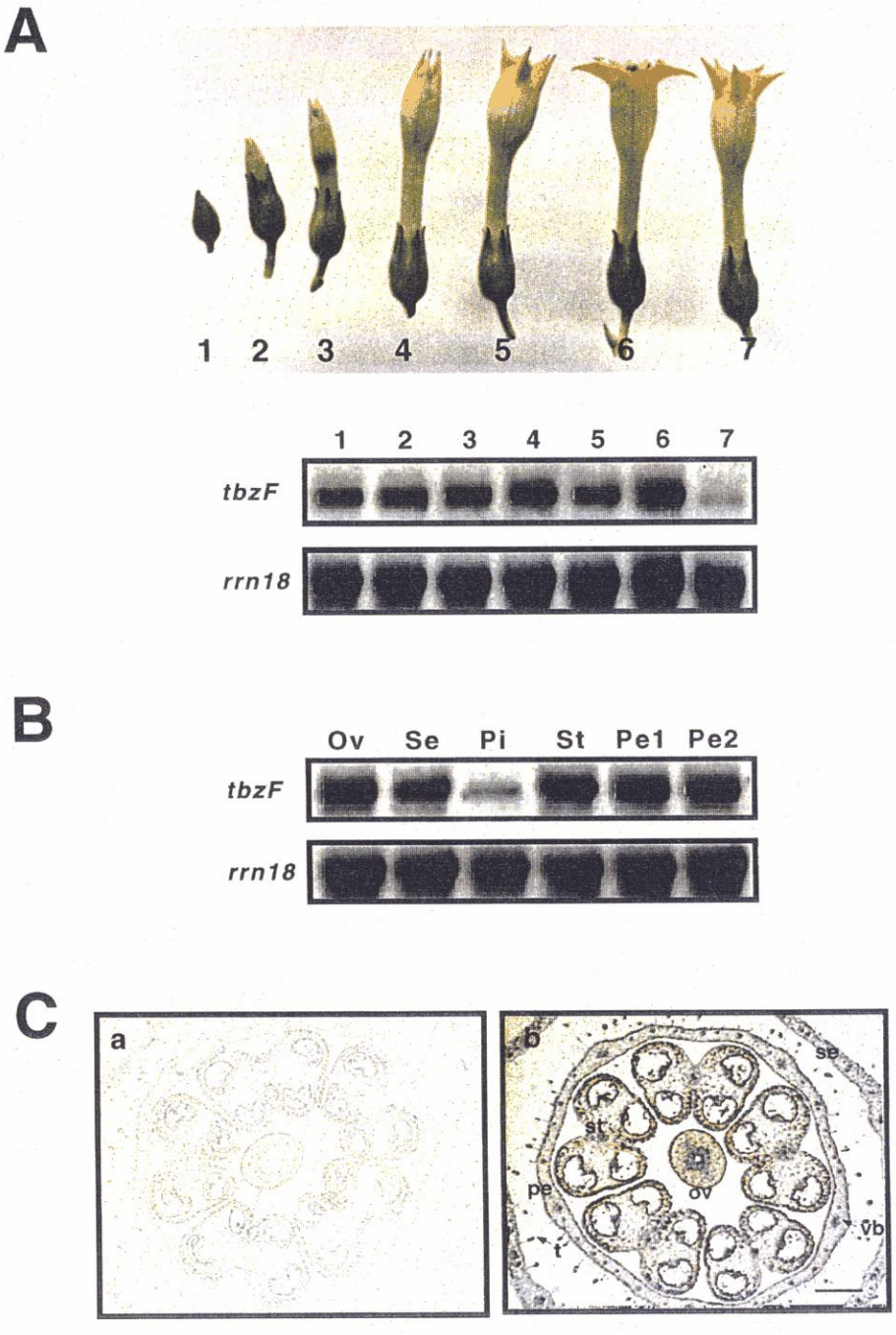


Fig. 25 Abundance of *tbzF*-transcripts along with flower development (**A**), in floral parts (**B**) and *tbzF* expression in flower determined by *in situ* hybridization (**C**). (**B**) ovule (O), sepal (Se), pistil (Pi), pink part of petal (Pe1) and lower white part of petal (Pe2). (**A** and **B**) The same filters were subsequently hybridized with the tobacco *rrn18* cDNA (*rrn18*, Ganal and Hemleben, 1986) to ensure equal sample loading. (**C**) horizontal-sections of flower bud at 3rd stage were hybridized with *tbzF* sense- (a) and antisense-probes (b). A bar in C is 20 µm.

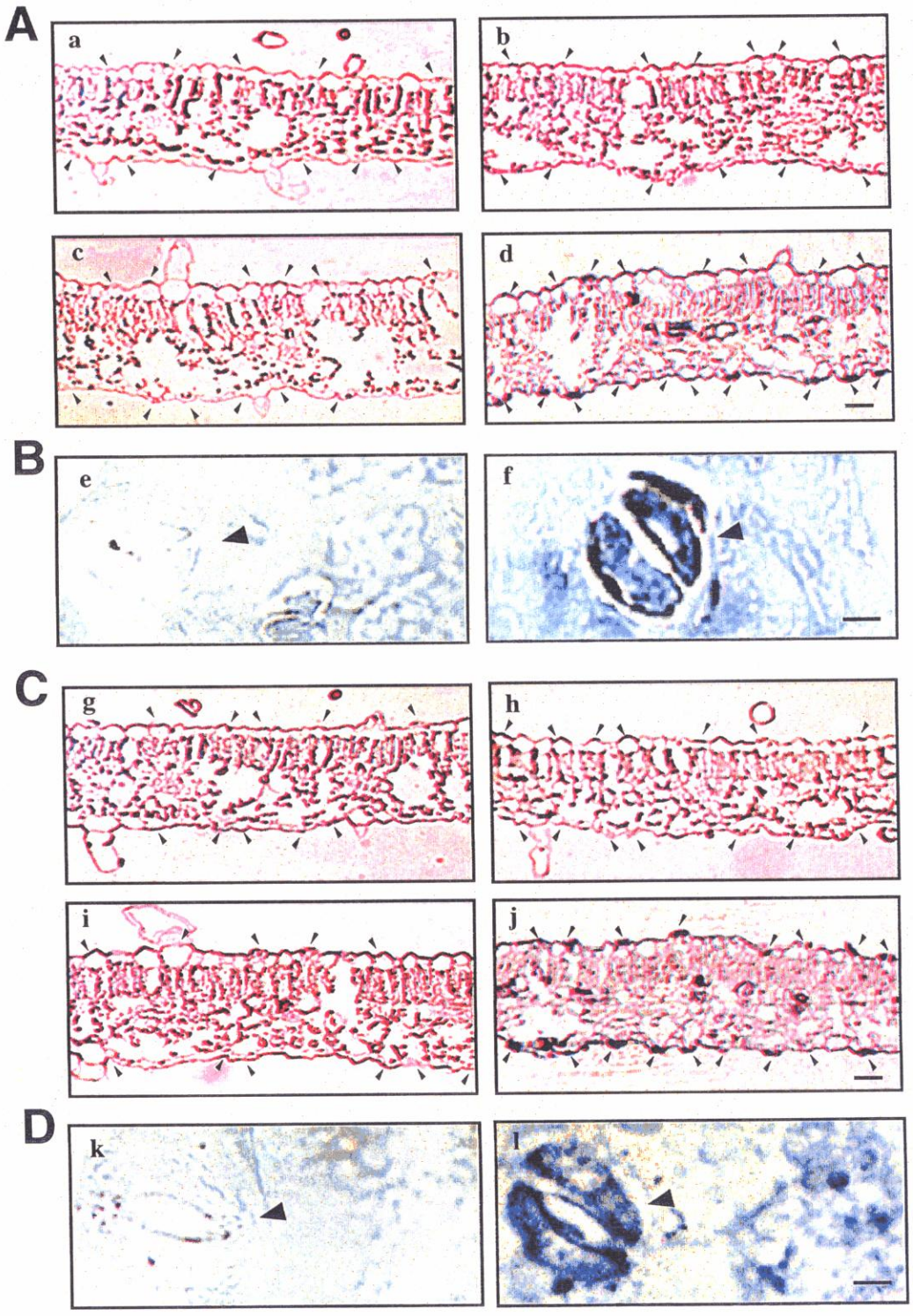


Fig. 26 *tbzF* and *tbz17* expression in leaves determined by *in situ* hybridization. Vertical sections of young leaves (**a, b, g** and **h**) and senescing leaves (**c, d, i** and **j**), and horizontal sections of senescing leaves (**e, f, k** and **l**) of tobacco plants were hybridizd with gene-specific sense- (**a, c, e, g, i** and **k**) and antisense-probes (**b, d, f, h, j** and **l**). of *tbzF* (**A** and **B**) and *tbz17* (**C** and **D**) genes. A bar corresponds to 10 µm (**A** and **C**) and 50 µm (**B** and **D**), respectively.

CHAPTER III

Promoter analysis of *tbzF*, a gene encoding a bZIP-type transcription factor, reveals distinct variation in *cis*-regions responsible for transcriptional activation between senescing leaves and flower buds in tobacco plants

Introduction

As described in Chapter II, I characterized two tobacco genes for bZIPs, *tbzF* and *tbz17*. The basic domain-leucine zipper proteins (bZIPs) contain a basic region and a leucine heptad repeat responsible for DNA binding and dimerization (Pysh et al., 1993; Hurst, 1994). They are considered to form homo- or heterodimers and to bind specific DNA sequences, thus functioning as transcription factors (Landschultz et al., 1988; Vinson et al., 1993; Yanagisawa, 1998). Most plant bZIPs bind to the ACGT core sequence, and have therefore been collectively referred to as ACGT elements (Weisshaar et al., 1991; Armstrong et al., 1992). However, bZIPs also require additional nucleotide sequences in the flanking regions for precise binding (Tabata et al., 1991; Schindler et al., 1992). In plants, more than 200 genes for bZIPs have so far been identified and the number may further increase in the future since, even in a single species, the genes constitute a superfamily. For example, Arabidopsis genome analysis revealed that this organism contains almost 100 forms (Riechmann and Ratcliffe, 2000).

Among plant genes for bZIPs, the *lip19* subfamily consists of at least 7 members, characterized by their specific response to cold stress (Aguan et al., 1993; Kusano et al., 1995; Ito et al., 1999; Yang et al., 2001). However, some members are also expressed in a cell-type-specific and/or a developmentally regulated fashion. For example, *A. majus 910* and *911* genes are expressed in flowers, predominantly in vascular tissues, carpels and anthers (Martínez-García et al., 1998). Maize OBF1, which was first identified as a binding factor to

the *ocs*-element in the promoter region of the octopine synthase gene of the *Agrobacterium tumefaciens* T-DNA (Singh et al., 1990), shows a gradient in transcript levels in developing leaves (Zhang et al., 1993). In the basal portions containing dividing and differentiating cells, the *OBF1* transcript level is 40-50 fold higher than in apical portions, where the cells are fully differentiated (Zhang et al., 1995). In the case of maize *mlip15,* transcripts accumulate with leaf aging (Berberich et al., 1999). These observations support the idea that the *lip19* members are regulated not only by environmental factors but also by developmental cues.

In this chapter, I examined whether or not *tbzF* is differentially regulated responding to physiological conditions. To this purpose, I analyzed promoter activities of *tbzF* by monitoring β-glucuronidase (GUS) activity. I addition, to identify promotion responsive regions, I transformed four deletion constructs into tobacco plants, and to used that the *cis*-element for senescence response is located on a different region from that for flower development.

Materials and methods

Plant growth and treatments

Tobacco plants were grown as described in Chapter II. Cold treatments were performed by transferring plants to a growth chamber set to 4°C for different periods of time under the light and photoperiodic conditions described above. Abscisic acid (ABA) was added to the 1/2 strength MS medium to a final concentration of 20 μM. Ethephon was applied to samples at the concentration of 1 mM for ethylene treatment. Leaves were collected at the 2nd and 3rd positions from the tops of two plants, frozen in liquid nitrogen and stored at -80°C until use.

Isolation of tbzF *genomic clones*

An *N. tabacum* genomic library was constructed and screened with *tbzF* cDNA (Yang et al., 2001) as the probe (Sambrook et al., 1989). The genomic sequence was determined by direct sequencing of a clone obtained by subcloning of a 9 kb *Bam*HI fragment. Since this fragment

lacked the 5' up-stream region of *tbzF*, TAIL-PCR was performed (Liu et al., 1993) using the following two *tbzF*-specific primers, *tbzF-sq1* (5'-GAGGAAATGAAAACACAAGC-3') and *tbzF-sq2* (5'-AGCGGGTTTATATAGGTGAG -3'). The products were subcloned into the pGEM-T easy vector (Promega), resulting in pGEM-*tbzF*. Double-stranded plasmids were sequenced with a ABI PRISM 310 sequencer using a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer, Norwalk, CT, U.S.A.) or with a Hitachi SQ-5500 sequencer using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham) (Sanger et al., 1977). Sequence data were assembled and analyzed with the SDC-GENETYX genetic information processing program (Lipman and Pearson, 1985; Software Development Co., Tokyo) and compared with sequences in non-redundant databases by the BLAST program (Altschul et al., 1990) on network servers.

Primer extension analysis

The Texas Red-labelled primer (5'-ACGTAGAACCAGTACAGGAAGACTACGG-3') (provided by Nippon Flour Mills Co. Ltd., Atsugi, Japan), complementary to nucleotides 171 to 144 of Fig. 27, was hybridized at 60°C for 1h with 10 μg of heat-denatured total RNA isolated by the AGPC method from senescing tobacco leaves. The annealed primer was extended with reverse transcriptase (Superscript II, GIBCO-BRL) in a 30 μl reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each of the dNTPs, and 10 mM DTT) at 42°C for 1h. The extended product was resolved by electrophoresis on a polyacrylamide gel containing 6% Long Ranger (Pharmacia)/6.1M urea in parallel with the sequencing ladder of the *tbzF* fragment obtained by TAIL-PCR starting with the same Texas Red-labelled primer.

Transgenic plants

Four *tbzF*-promoter *GUS* chimeric genes were made by ligating 5' *tbzF* sequences into the promoter-less GUS expression vector pBI101.1 (Clontech, Palo Alto, CA). A -1740 to +344bp fragment of the *tbzF* gene was engineered to have *Hin*dIII and *Bam*HI sites, and ligated into *Hin*dIII and *Bam*HI sites of the pBI101.1. For the three deletion constructs, the

-1220 to +344, -720 to +344 and -220 to +344 fragments of the *tbzF* gene were cloned into *Hin*dIII and *Bam*HI sites of the pBI101.1 as described above. Transformation of tobacco (*N. tabacum* L. cv. Xanthi) was performed as detailed earlier (Nakano et al., 2000). The *Agrobacterium tumefaciens* LBA4404 stain containing the four constructs were inoculated to tobacco (*N. tabacum* cv. Xanthi) leaf disks. Once rooted on a selective medium (50 $\mu g/ml^{-1}$ Kanamycin), shoots that were 5-10 cm in height were transferred to peat compost and grown in a greenhouse. T1 progeny were sown on 1/2 MS agar containing 50 $\mu g/ml^{-1}$ Kanamycin. The presence of a transgene in the plants was confirmed by genomic PCR with appropriate primers.

RNA isolation and northern hybridization

Extraction of total RNA and RNA blot analysis were carried out as described in Chapter I.

Chlorophyll assays

Chlorophyll contents were measured as described in Chapter I.

Assays of GUS activity and histochemical staining

GUS activity in whole extracts was determined essentially as described (Jefferson, 1987). Histochemical localization of GUS activity was performed by incubating various tissues of transgenic plants in 5-bromo-4-chloro-3-indolyl-glucuronic acid solution at 37°C for 3h to overnight until the blue staining had reached sufficient intensity. Samples were fixed with formaldehyde:acetic acid:40% (v/v) ethanol (5:5:90), and then chlorophyll was removed by passage through a 50% to 100% ethanol series. To allow better penetration of the substrate, a vacuum was applied for 30s. Staining, tissue embedding, and hand-cut sectioning were performed as described previously (Mandel et al., 1995).

Results

Isolation of the tbzF *promoter*

By a combination of genomic library screening and TAIL-PCR, a 1740 bp DNA fragment was finally isolated (Fig. 27). The initiation site of transcription was determined by primer extension analysis, and designated +1 (Fig. 28). The initiation codon for translation is located at +344 nucleotides downstream from the initiation site of transcription. A typical TATA-box is located at position -52 (TAATATTT). The isolated fragment was analyzed for putative *cis*-acting elements by the PLACE Program (Higo et al., 1999). The most prominent matches identified include those to the consensus binding sites for bZIP (ACGT), for TAAAG motifs (TAAAG) (Plesch et al., 2001), and for pollen-specific regulatory elements (AAATGA) (Weterings et al., 1995) (Fig. 27). In addition, G-box motifs (CACGTG) were found similar to the G-box-like sequence (TACGTG) (Schindler et al., 1992; Shinozaki and Yamaguchi-Shinozaki, 1997).

Expression of the GUS *reporter gene*

The promoter region with 1740 bp was ligated to the *GUS* reporter gene in a translationally fused manner, and introduced into tobacco plants. Among 30 regenerated transgenic plants, 10 healthy specimens were selected and assayed for promoter activity using materials including flowers, leaves at different positions and roots. To evaluate the degree of aging, chlorophyll content in each sample was estimated, this showing a proportional decrease with increase in the age (Fig. 29A). When GUS activity was measured in these samples in the absence of external stress, a clear positive relationship was observed, i.e., the older were the leaves, the higher the GUS activity (Fig. 29B). In addition, strong activity was observed in flower buds, equivalent to that in the oldest leaves (Fig. 29B). This was confirmed by RNA gel blot analysis, showing *GUS* transcripts to proportionally increase in line with enzymatic activity (Fig. 29C). As endogenous *tbzF* transcripts showed exactly the same accumulation profile as GUS transcripts (Fig. 29C), it was concluded that the reporter system employed faithfully reflects the behavior of *tbzF* gene *in planta*, and that the isolated 1740 bp region covers all the elements necessary for promoter activity.

Tissue specificity of the tbzF *promoter*

Transgenic tobacco plants carrying the reporter *GUS* gene fused to the 1740 bp promoter were grown in plant boxes, and histochemical assayed by GUS staining. First, tissue-specific activity was monitored (Fig. 30A). In young leaves, expression was essentially not detectable (Fig. 30A, a-c). In contrast, senescing leaves were stained deeply in guard cells, vascular tissues and trichomes (Fig. 30A, d-f). This was particularly clear in peeled epidermis (Fig. 30A, b and e).

In 8-week old transgeinc tobacco plants, expression of GUS was observed in the flowers (Fig. 30B). Strong GUS activity was observed in anthers, filaments, ovaries and sepals, but not in stigmas or styles (Fig. 30B, g-j). Again, guard cells of the petals, trichomes and veins were the main source of expression (Fig. 30B, k and l).

Effects of Cold, ABA and Ethylene

Since senescence is closely associated with the phytohormones cold, ABA and ethylene, the transgenic plants were treated with these compounds and the reporter activity assayed (Oh et al., 1996). First, RNA gel blot analysis was performed with *tbzF* and *GUS* probes (Fig. 31A). The probe specific to *tbzF* was a DNA fragment corresponding to the 3' untranslated region of the *tbzF* cDNA. Transcripts of *tbzF* were induced within 24 h after treatment with cold, ABA and ethephon, and were further detectable for up to 2 days (Fig. 31A). Transcripts of *GUS* were also induced, showing the same profile as for endogenous *tbzF* (Fig. 31A). Then, enzymatic activity of GUS was examined with the same samples used for RNA blot analyses. Treatment with cold, ABA and ethephon induced 5-fold and 3-fold higher GUS activity, respectively, in comparison with untreated samples (Fig. 31B). Then, effects of cold, ABA and ethylene were examined using young seedlings. While untreated control samples showed no staining (Fig. 32, a-c), cold-, ABA- and ethylene-treated seedlings demonstrated high GUS activity in detached leaves, stems and roots (Fig. 32, d-l). GUS activity was mainly observed in guard cells in detached leaves, (Fig. 32, d, g and j), and in vascular tissues of the transition zone of stems (Fig. 32, e, h and k). Elongation and transition zones of roots were also actively stained (Fig. 32, f, i and l). These experiments confirmed that the isolated

promoter region contains *cis*-acting elements that are necessary for the cold, ABA and ethylene response.

Deletion analysis

In order to identify the responsible regions for promoter activity, deletion constructs of 1740 bp fragment was made and fused to the *GUS* reporter. One or more approximately 500 bp sequences were deleted from the original, yielding promoter fragments of 1220, 720 and 220 bp (Fig. 33). They were fused to *GUS* reporter gene, and subsequently introduced into tobacco plants. General survey of GUS activity in different organs of R_0 transgenic tobacco plant. Results of a general comparison are shown in Fig. 34. Ten stable transformants were selected for each deletion construct, and assayed for enzymatic activity in senescing leaves and flower buds. In senescing leaves, transformants carrying -1740 and -1220 promoters showed more than 3-fold the basal activity in young leaves (Fig. 35A). However, clear decline to one third was evident in transformants carrying -720 and -220 promoters (Fig. 35A). In flower buds, the –1740 and -1220 transformants caused similar levels of activity as the original, over 3-fold that of young leaves. In addition, the same activity was apparent with the -720 construct, in sharp contrast to the case with senescence and phytohormone treatment (Fig. 35A). Total RNA was extracted from the 4 constructs, and RNA blot analyses were then performed. Transcripts of the reporter *GUS* accumulated abundantly in senescing leaves of transformants with -1740 and -1220 promoters, but at much lower levels with the -720 and -220 promoters (Fig. 35B). In flower buds, however, transcript accumulation was equally high in transformants carrying -1740, -1220 and -720 promoters and decrease was only observed with the -220 construct (Fig. 35B).

Ten stable transformants were selected for each deletion construct, and assayed for enzymatic activity under various conditions. The observation that exposure to cold induced a gradient decrease in expression of GUS activity as the deletion increased (Fig. 36A). When young leaves of transformants with -1740 and -1220 promoters were treated with ABA and ethephon, GUS activity increased to over 3-fold that of untreated control samples. This level was decreased to less than half in -720 and -220 transformants, showing the same pattern as in

senescing leaves (Fig. 36B and 36C).

These results were thus consistent with the GUS activity assay, again indicating the presence of different *cis*-element(s) responsive with regard to senescence and flower development. At least three distinct regions are present: the proximal 220 bp serving as the basic region which renders low but consistent activity; the middle 500 bp region for flower-specific expression; and the far -500 bp region for senescence-asociated expression. Each contains particular domains, including pollen-specific and Q-elements, and G-box and TAAAG motifs, in the flower- and senescence-specific regions, respectively (Fig. 37).

Discussion

As described in chapter II, I described *tbzF* transcripts to accumulate not only upon cold, ABA and ethylene treatments, but also during leaf senescence and flower development in wild-type tobacco plants. Furthermore, *in situ* hybridization showed *tbzF* transcripts to be predominantly expressed in guard cells of senescing leaves, vascular tissues, trichomes, and in petals and sepals of flowers. Further analysis in the present study confirmed these findings for expression of *tbzF* gene using a promoter-*GUS* reporter system, and in addition demonstrated the existence of distinct *cis*-elements for promoter response regarding senescence and flower development.

Only few promoters have so far been studied for specific gene expression in guard cells (Müller-Röber et al., 1994; Nakamura et al., 1995). One example is the promoter of the guard cell-specific *KST1* gene from potato (Müller-Röber et al., 1995), in which a *cis*-acting element (TAAAG) was identified (Plesch et al., 2000), featuring the core binding sequence of Dof proteins (Yanagisawa and Schmidt, 1999). In the ABREs of several ABA-responsive genes, including the maize *rab 28* and wheat *Em1a* genes (Pla et al., 1993; Marcotte et al., 1989), a so-called G-box-related sequence (TACGTG) has been described. G-box elements comprise a family of *cis*-acting sequences active in expression of genes that respond to cold, ABA and drought (Schindler et al., 1992; Nordin et al., 1993). The present search of the

promoter region between -720 and -1740, where senescence and/or guard cell specific elements may reside, demonstrated four Dof protein binding sites and one G-box element (Fig. 37). Since such elements are lacking in the +1 ~ -720 promoter region, and since deletion constructs (-720 and -220) lost responses to senescence, ABA and ethylene, it was conceivable that they indeed serve as *cis*-elements for *tbzF* expression during senescence. However, among four TAAAG elements in the -1740 construct, the first two in the near proximal to the coding region appear to be sufficient, conferring full expression of the reporter gene.

To date, flower specific promoter analysis has identified at least two distinct elements: the pollen-box (Twell et al., 1990, 1991; Eyal et al., 1995; Bate and Rwell, 1998) and the Q-element (Hamilton et al., 1998). The pollen box is AAATGA, which is frequently observed in the regulatory regions of late pollen-specific genes (Mitsuda et al., 2001). It is capable of driving pollen-specific gene expression, independent of the orientation (Weterings et al., 1995). The Q-element (AGTCA) was first identified as a 'quantitative element' in the regulatory region of the pollen-specific maize *ZM13* gene (Hamilton et al., 1998). In the *tbzF* promoter, flower-specific *cis*-elements appear to be present in the region region between -220 and -720. Within this 500 bp sequence, we identified four pollen-specific elements (TCATTT), and one Q-element (AGTCA) (Fig. 37), their total numbers in the 1740 bp promoter region being five and four, respectively. The results suggest that *tbzF* possesses at least two distinct promoter regions: one for senescence-response at the distal half, and the other for flower development in the proximal half (Fig. 37). In addition, the most proximal ~220 bp region appears to serve as a common basic promoter for *tbzF* transcription (Fig. 37). Taken together, our findings point to plants utilizing a gene for multiple purposes by converging different signals upon distinct regions of its promoter sequence.

Finally, it should be mentioned that exposure to cold induced a gradient decrease in expression of GUS activity with increase in the magnitude of the deletion (Fig. 36A). This was in clear contrast to the case with senescence and flower development, and suggests the presence of other *cis*-elements necessary for a cold-response. In fact, I have already revealed the existence of many cold-related *cis*-elements such as a LTRE/DRE/C-repeat core sequence

(CCTAC), a MYC-like sequence (CATATG) and a MYB-like sequence (ACCTACC) in the 1740 bp promoter region (Fig. 27). All of these are reported to be essential for low-temperature and drought (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994; Medina et al., 2001), dehydration and ABA responses (Williams et al., 1992; Abe et al., 1997; Busk and Pages, 1998). Currently, however, my data are too preliminary to allow a definitive conclusion to be drawn, and further analysis, utilizing more finely deleted constructs as well as point-mutated sequences, is awaited.

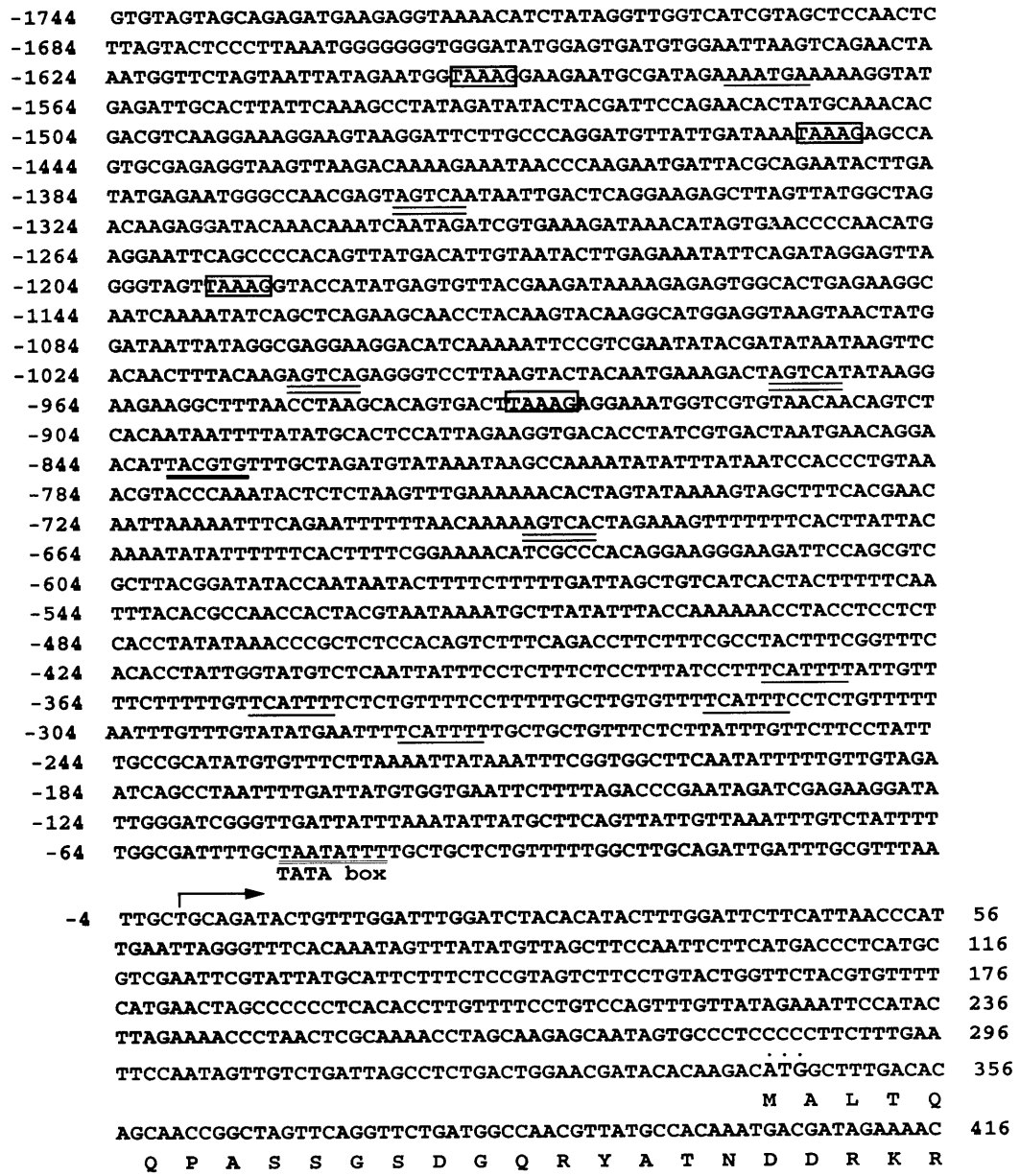


Fig. 27 Nucleotide sequence of the *tbzF* promoter region. The transcription initiation site is designated as +1 and indicated with an arrow. The initiation ATG codon and putative TATA-box are double underlined and the G-box-related sequence (TACGTG) is shown in bold underlined. The Dof protein binding site (TAAAG) is indicated by an open box and the pollen-specific regulatory elements (AAATGA and TCATTT) are underlined. The Q-element (AGTCA) is also double underlined.

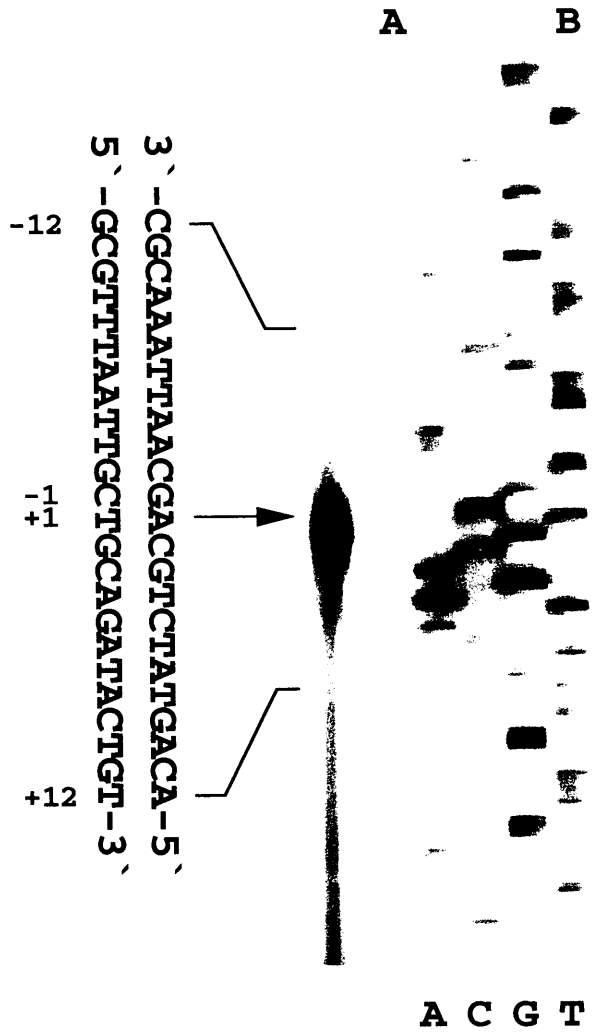


Fig. 28 A primer extension analysis to determine a transcriptional start site of *tbzF* gene. A Texas-Red labelled primer (see Materials and Methods) was hybridized with 10 µg of total RNA from senescing leaves and extended by reverse transcriptase. Sequencing reaction for the TAIL-PCR fragment was carried out with the same labelled primer. The extended product (marked with **A**) was electrophoresed in parallel with the sequencing products (marked with **B**) on 6% Long Ranger/ 6.1 M urea gel. The major extended product was indicated by arrow.

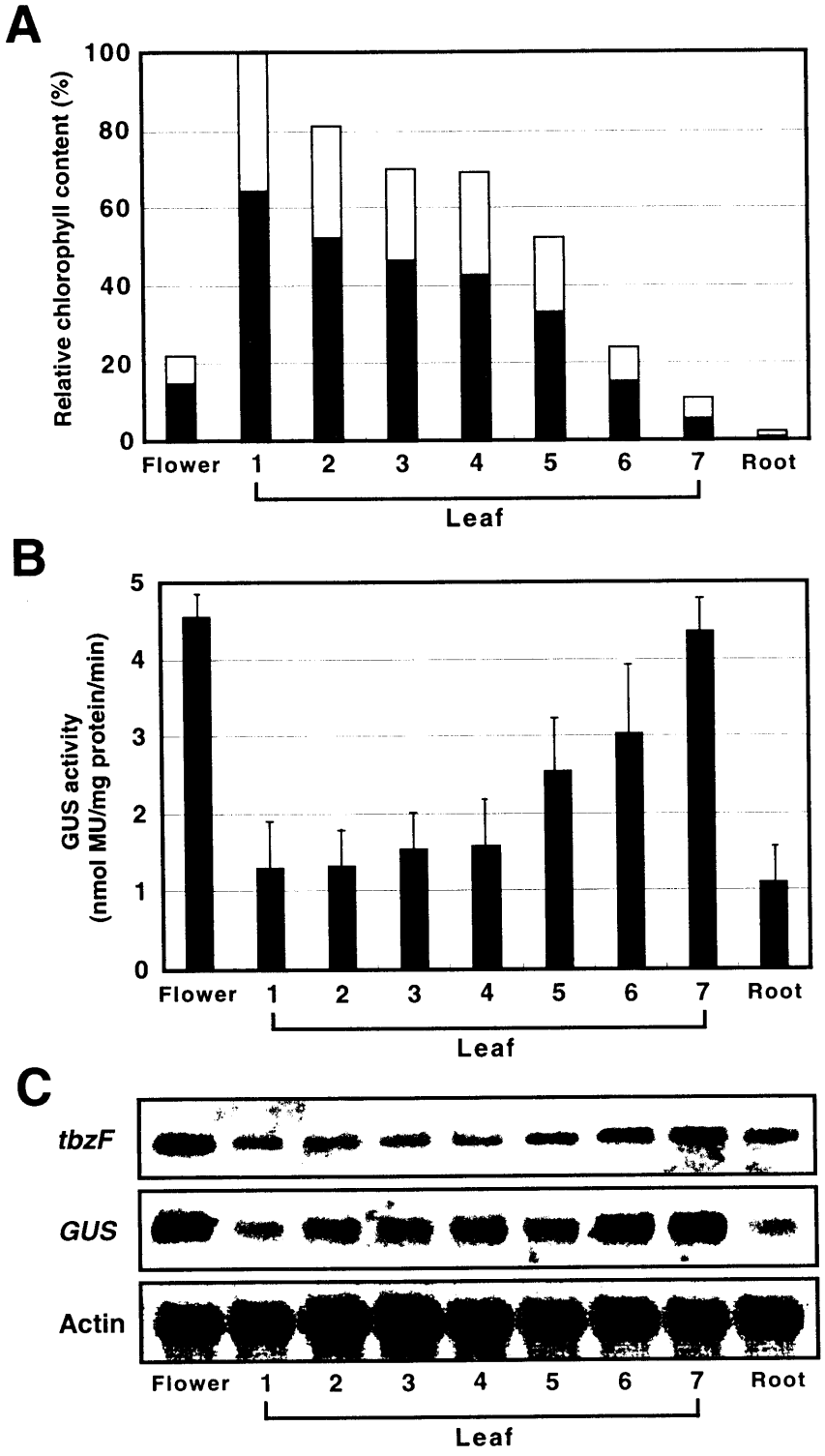


Fig. 29 Organ-specific expression of the *tbzF-1740* promoter-*GUS* chimeric gene. (**A**) Chlorophyll content. Chlorophylls a (filled bar) and b (open bar) were measured as described in the Materials and methods. (**B**) GUS activity in different tissues. The values are averages of data obtained from ten independent transformant lines. Bars indicate standard deviation (SE). (**C**) RNA blot hybridization analysis. Aliquots of 20 μg total RNA per lane were fractionated by electrophoresis, transferred to a membrane and subjected to hybridization with probes for the *tbzF* or *GUS* genes. The same filter was subsequently hybridized with the tobacco actin cDNA as a sample loading control.

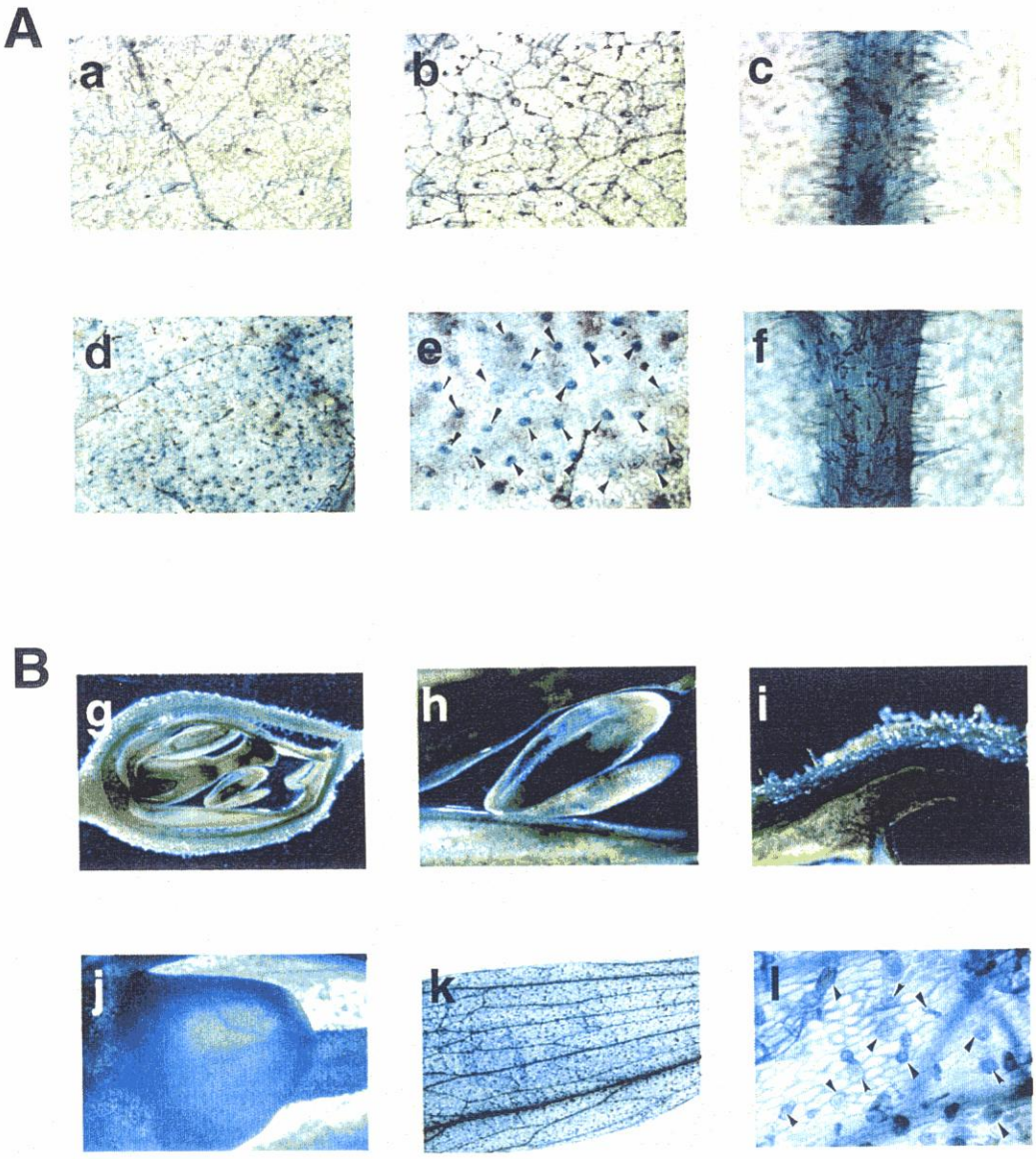


Fig. 30 Identification of GUS activity by histochemical staining in tobacco plants expressing the *tbzF-1740* promoter-*GUS* chimeric gene. (**A**) Senescence-specific expression. GUS is expressed not in young leaves (**a** through **c**), but in senescing leaves (**d** through **f**). Samples are from leaf (**a**, **d**), peeled epidermal strip (**b**, **e**) and stem (**c**, **f**). Note the high expression in stomatal guard cells (**e**; indicated by arrows) and in trichomes of an epidermal peel (**f**). (**B**) Flower-specific expression in cross-sections of a flower bud (**g**), pollen grains and stigmas of a flower (**h**), silique in the petals (**i**), a longitudinal section of an ovule (**j**), a longitudinal section of the guard cells of petals (**k**) and a cross-section of a petal (**l**). Arrows indicate guard cells.

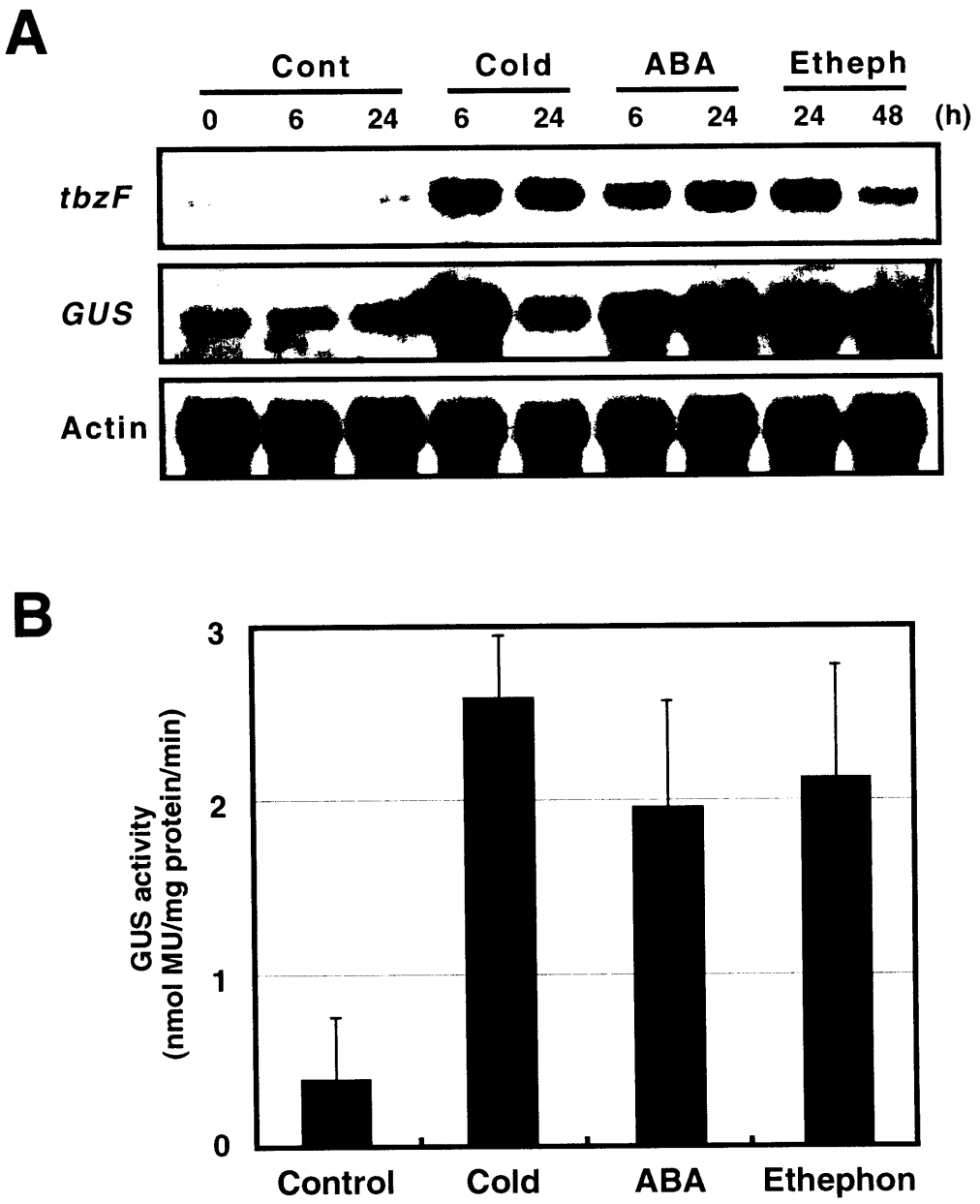


Fig. 31 Expression of the *tbzF-1740* promoter-*GUS* chimeric gene in response to ABA and ethylene treatments. (**A**) RNA blot hybridization analysis. Total RNAs were extracted from transgenic tobacco leaves untreated (Control), exposed to 4°C for 24 h (Cold), treated with 20 μM ABA (ABA) or 1 mM ethephon (Ethephon), and 20 μg aliquots were subjected to RNA blot hybridization analysis as described in the legend for Fig. 29. (**B**) GUS activity assay. Ten-day-old seedlings untreated (Control), exposed to 4°C for 24 h (Cold), treated for 24 h with 20 μM ABA (ABA) or 1 mM ethephon (Ethephon), were sampled and assayed for GUS activity. Mean values ($P < 0.05$) are expressed in nmol MU per mg protein per minute with data from 10 independent lines. Bars indicate standard deviation (SE).

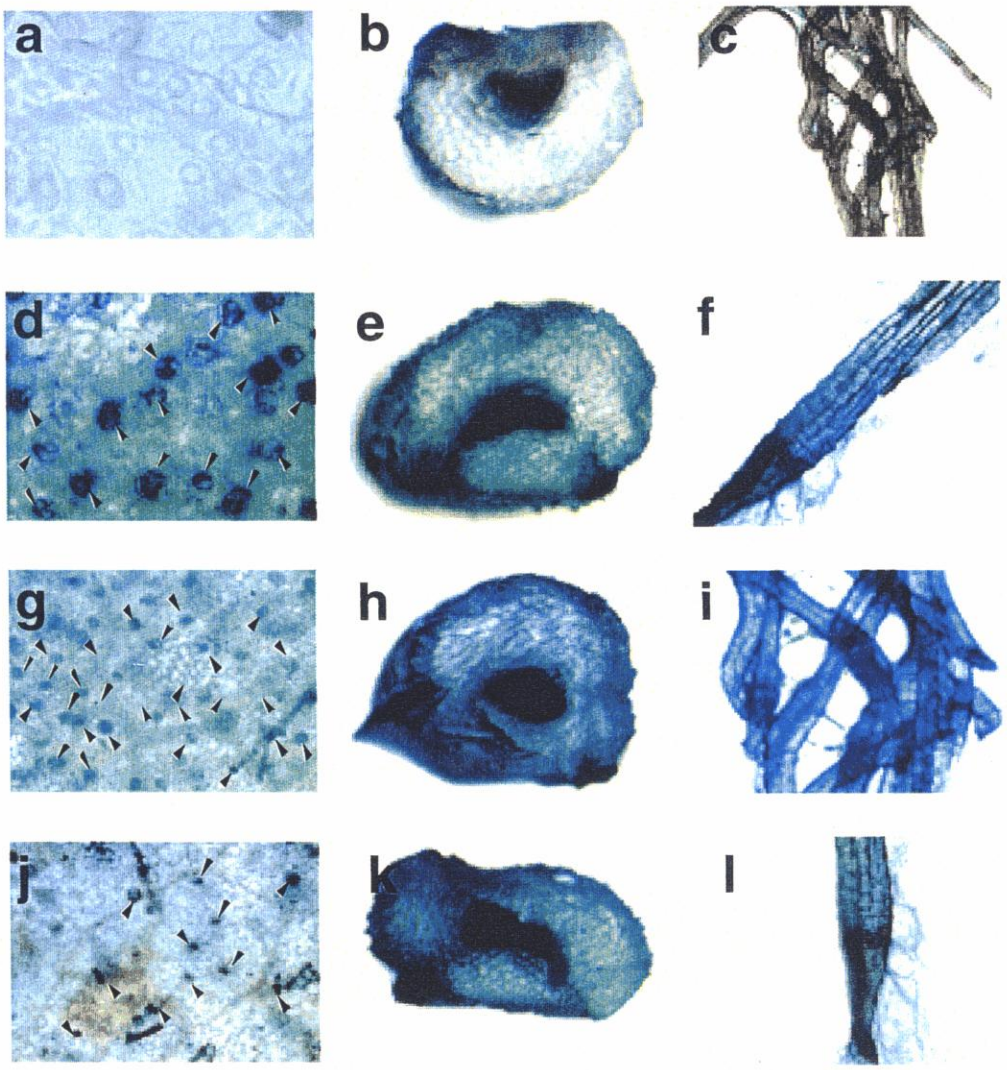


Fig. 32 Identification of GUS activity by histochemical staining in tobacco plants expressing the *tbzF-1740* promoter-*GUS* chimeric gene. Effects of cold, ABA and ethylene. GUS is lacking in untreated samples (**a** through **c**), but is expressed in cold- (**d** through **f**), ABA- (**g** through **i**) and ethephon-treated samples at 24 h (**j** through **l**). Samples are from leaf (**a**, **d**, **g** and **j**), stem (**b**, **e**, **h** and **k**) and root (**c**, **f**, **i** and **l**). Arrows indicate stomatal guard cells with strong GUS expression (**d**, **g** and **j**).

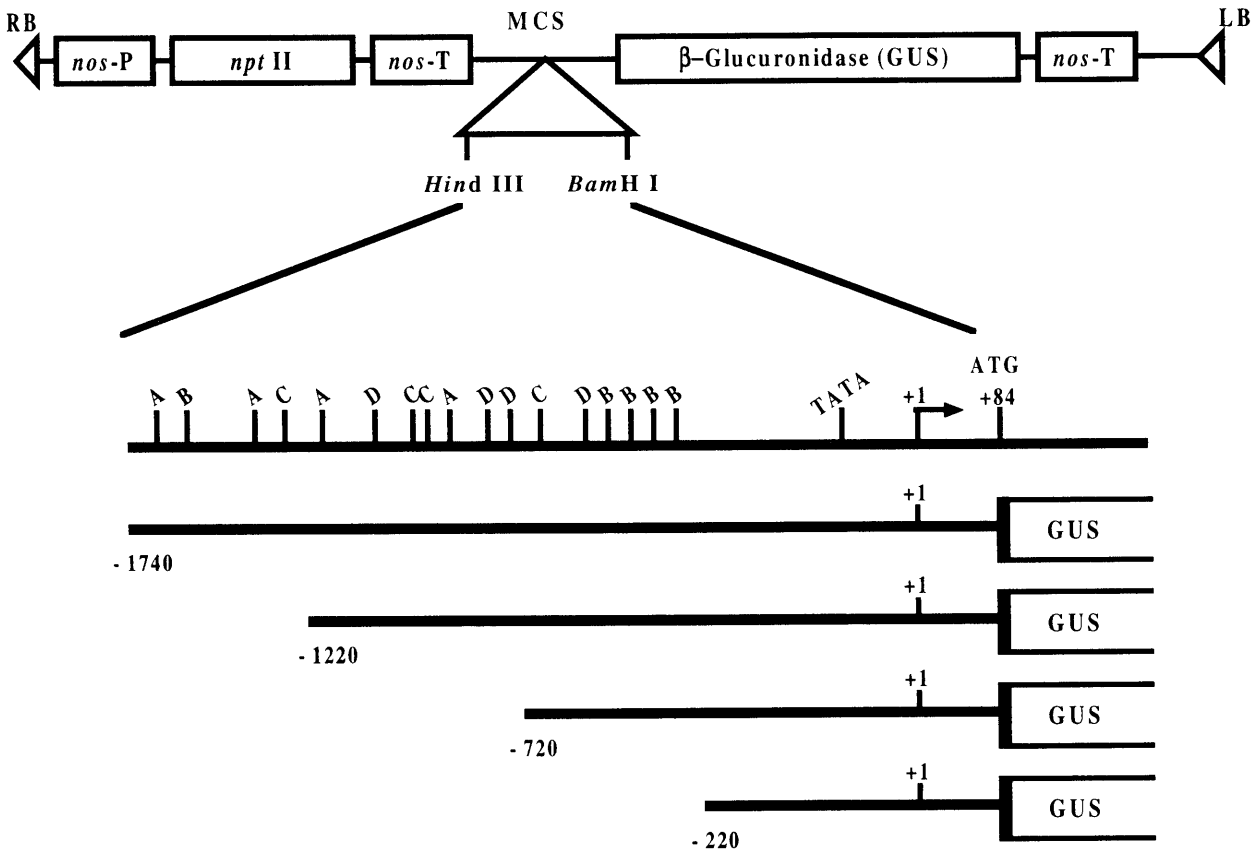


Fig. 33 Regions of *tbzF* included *tbzF-gus* fusions. The *tbzF* sequences that were fused to *gus* are indicated. the numbers refer to the nucleotide sequence pregented in Fig. 27. Four *tbzF*-promoter *GUS* chimeric genes were made by ligating 5' *tbzF* sequences into the promoter-less GUS expression vector pBI101.1. One or more approximately 500 bp sequences were deleted from the original, yielding promoter fragments of 1220, 720 and 220 bp. The locations of the repeated motifs marked with A, B, C and D represent sites similar to the putative TAAAG elements (TAAAG), pollen-specific (AAAATGA or TCATTTT), Q-element (AGTCA) and G-box-like sequence (TACGTG), respectively. Putative TATA-box (TATA), transcription site (arrow) and translation initiation site (ATG) are shown.

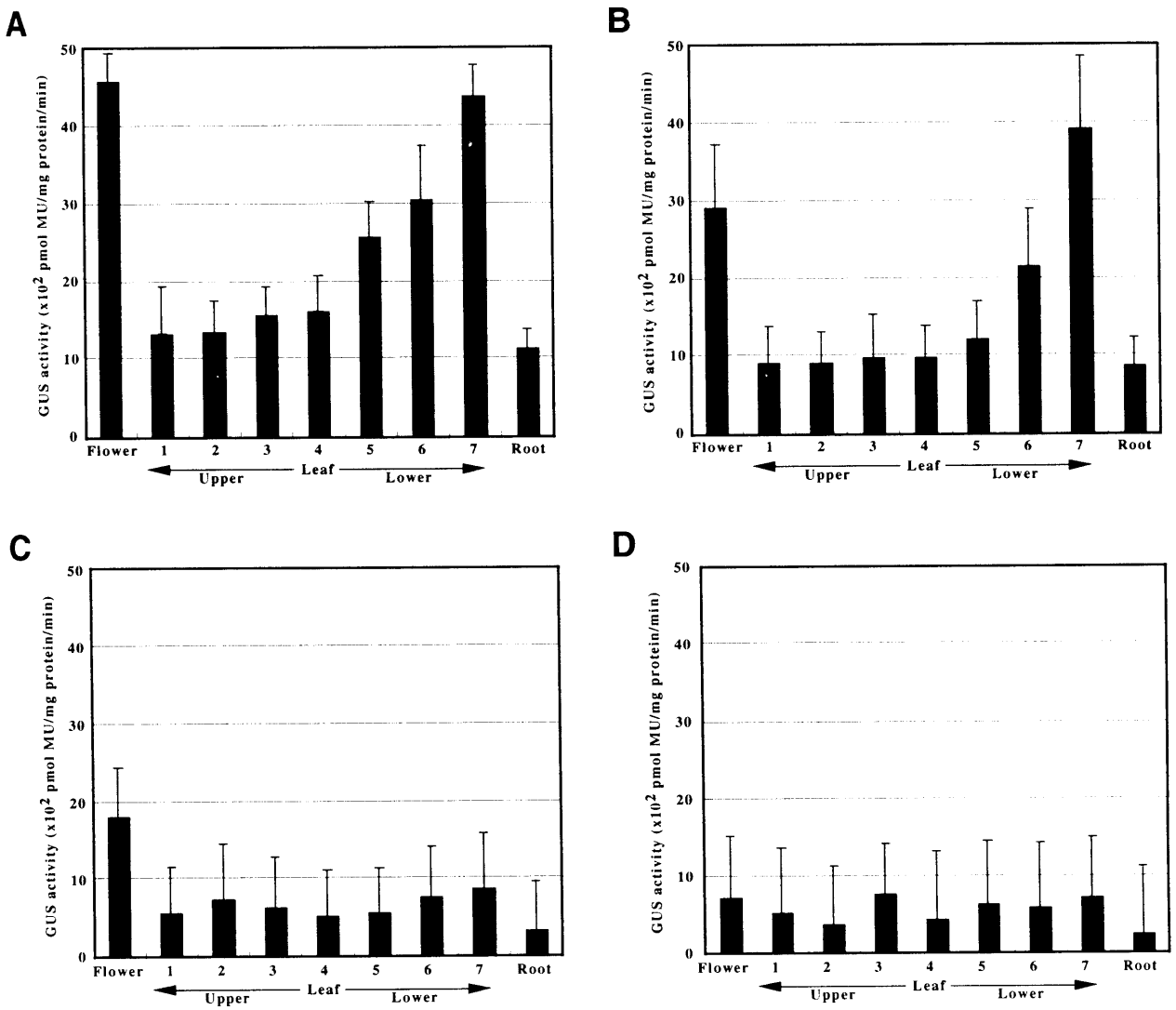


Fig. 34 Deletion analysis of the GUS activity in different organs of R_0 transgenic tobacco plants. The values are averages of data obtained from ten independent transformant lines. Bars indicate standard deviation (SE). Extracts was prepared from various sources of individual transgenic plants and used for GUS and protein assays. Data from one representative plant of each construct were plotted: (**A**) tbzF-1740, (**B**) tbzF-1220, (**C**) tbzF-720 and (**D**) tbzF-220.

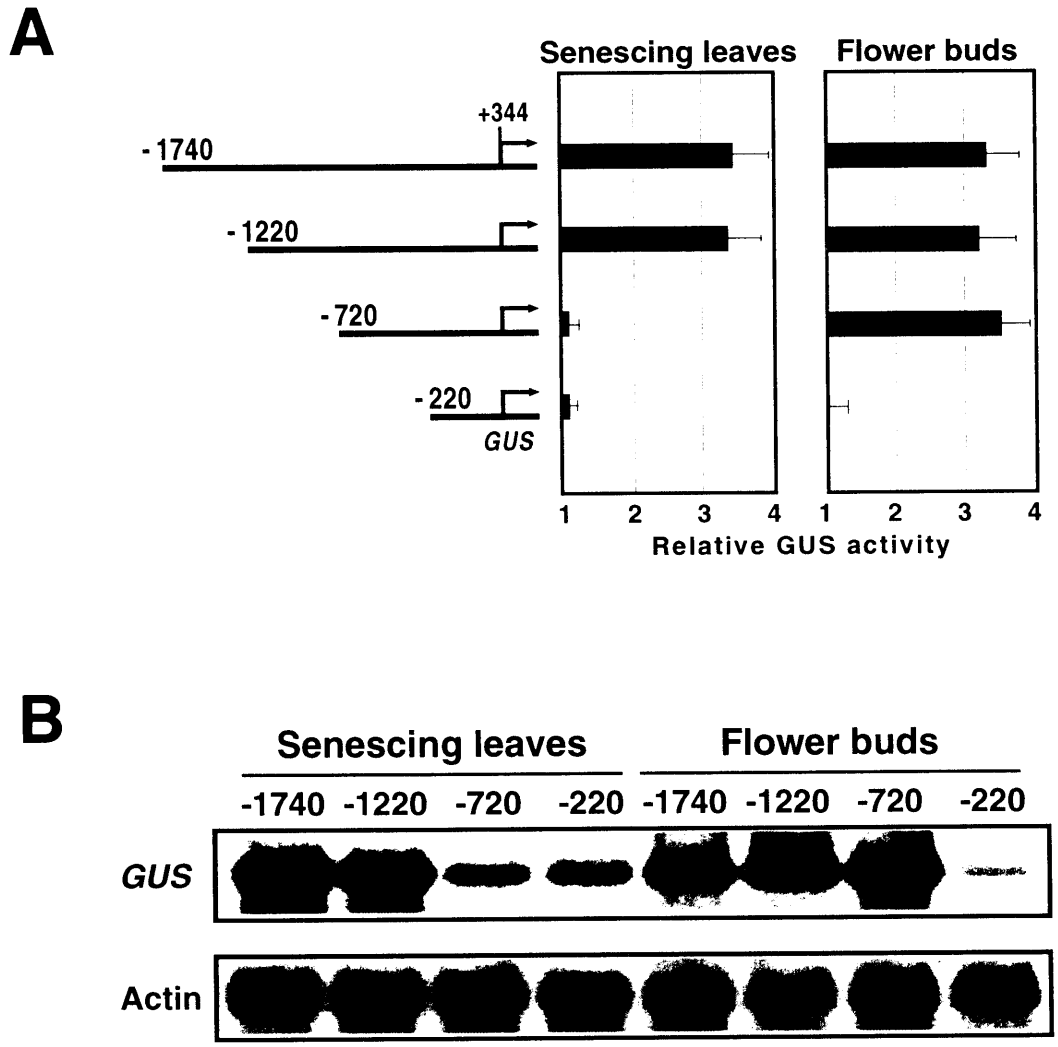


Fig. 35 Tissue-specific expression of the *tbzF-GUS* chimeric gene in tobacco plants. (**A**) Quantitative analysis of GUS activities driven by deleted promoters. GUS activity was measured in 10 independent transgenic plants for each construct. The ratio of the GUS activity in senescing leaves/young leaves, and in flower/young leaves was set as 1.0 to calculate relative values for senescencing leaves and flower buds, respectively. (**B**) RNA-blot analysis. RNA samples were extracted from senescencing leaves and flowers of four independent transgenic plants of the indicated deletion lines. Hybridization was conducted as described in the legend for Fig. 29.

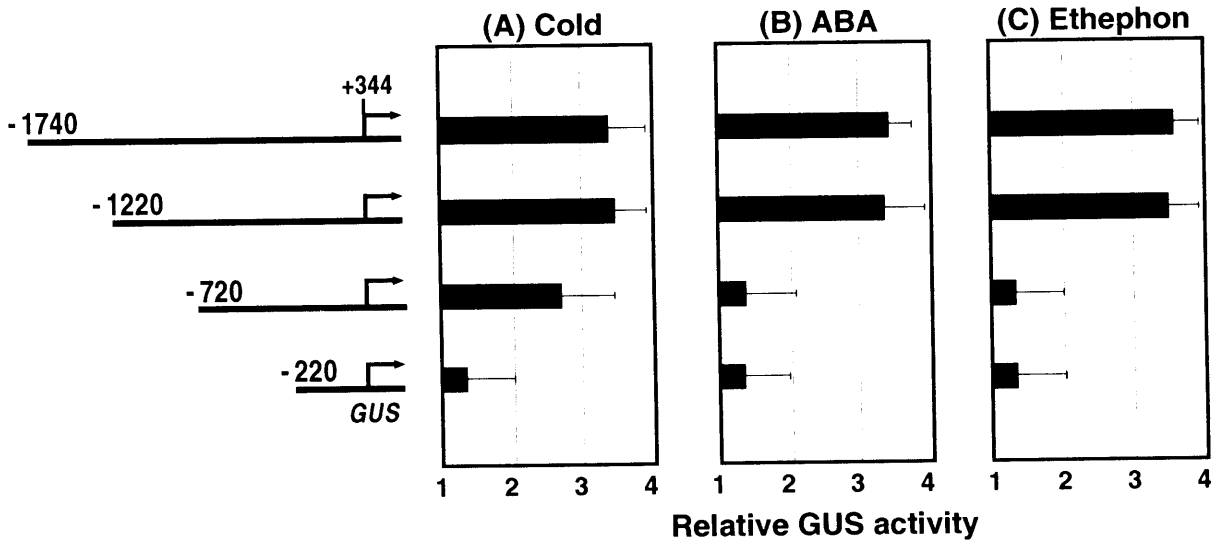


Fig. 36 Quantitative analysis of the *tbzF-GUS* chimeric gene in control and various treated tobacco plants. Quantitative analysis of GUS activities driven by deleted promoters. GUS activity was measured in 10 independent transgenic plants for each construct. Two-week-old seedlings grown at 25°C, exposed to 4°C for 24 h, treated with 20 μM ABA, or with 1 mM ethephon. The ratio of the GUS activity in treated leaves/untreated leaves was set as 1.0 to calculate relative values for cold, ABA and ethephon, respectively (**A**, **B** and **C**).

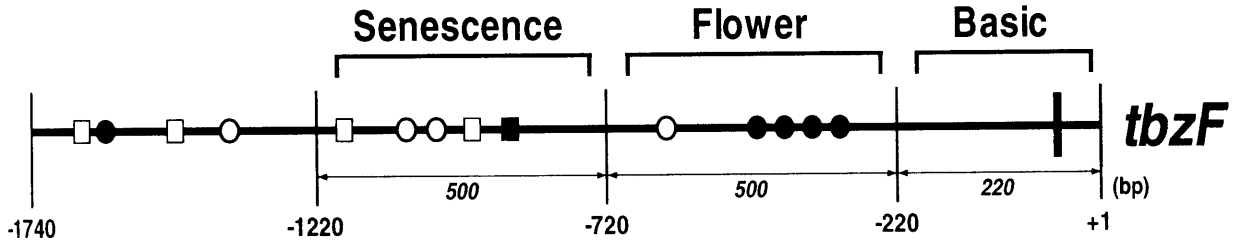


Fig. 37 Tentative regions responsible for senescence- and flower-specific expression of *tbzF*. Three regions are shown by brackets with bold numbers indicating nucleotide positions from the translation initiation site (+1). Numbers in italic indicate the nucleotide length of each region. Putative *cis*-acting elements are dof (open box), a G-box-like sequence (closed box), a Q-element (open circle), pollen-specific (closed circle) and a TATA-box (vertical bar).

CHAPTER IV

Isolation of TBZF interacting factors by yeast two-hybrid system

Introduction

The bZIP transcription factors form a large diverse family in plants. More than 200 genes for bZIPs have so far been identified and the number may further increase in future. Even in a single species, the genes constitute a superfamily. For example, Arabidopsis genome analysis revealed that this organism contains almost 100 forms (Riechmann and Ratcliffe, 2000). The bZIPs are caracterised by basic region establishing sequence-specific DNA binding and an adjacent haptad leucine repeats form two parallel a helices arranged as a coiled-coil and enabling dimerization of two protein which determine the orientation of the basic domains within the major groove of the DNA (Landschultz et al., 1988; Vinson et al., 1993).

The functional role of bZIP transcription factors has been linked to a large diversity of biological aspects, for example response to light (Oyama et al., 1997; Schulze-Lefert et al., 1989), hormones (Fukazawa et al., 2000), biotic (Zhou et al., 2000) and abiotic stresses (Aguan et al., 1993; Ito et al., 1999), as well as cell proliferation (Mikami et al., 1995) and developmental processes (Chuang et al., 1999; Yang et al., 2001). However, molecular mechanism, in which bZIPs function to regulate gene expression is largely not clear. Two features must be clarified; identification of protein complex in which bZIP is involved and participates in 'on-off' of gene expression, and identification of target genes.

In this chapter, I describe identification of genes encoding proteins that interact with TBZF by yeast two-hybrid screening. Among several clones, one particular clone was found to encode a protein that predominantly interacts with TBZF, and designated *NtTIP1* (*N.tabacum* TBZF interacting protein1). *NtTIP1* encodes a 12.5-kDa basic protein, which is

localized in the nucleus, and is expressed during senesscence and fruit ripening and upon water deficit.

Materials and methods

Plant materials and chemical treatments

Tobacco plants were grown as described in Chapter II. Seedlings, grown on organ plants were carefully removed, transferred to liquid 1/2 strength MS medium and maintained for another 2 days before the start of any treatment. In the case of hormone treatments, abscisic acid (ABA, 20 μM), 6-benzylaminopurine (BA, 20 μM), (-) jasmonic acid (JA, 20 μM) or indole-3-acetic acid (IAA, 20 μM) were added to the 1/2 strength MS medium. For salt treatment, NaCl was added from a stock solution to the medium to give a final concentration of 0.2 M. For ethylene treatment, plants were exposed to 10 μl/l ethylene gas in special glass containers. At each time point, 2nd and 3rd leaves from the top of plants were harvested, frozen in liquid nitrogen and stored at -80°C until use.

Construction of plasmids for two-hybrid screening

The expression cDNA libraries were constructed from mRNA isolated from tobacco leaves using Uni-ZAP XR and HybriZAP vectors (Stratagene; Moon et al., 1999). Extraction of total RNA from tobacco seedlings was performed as described above. Poly $(A)^+$ mRNA was isolated according to the protocol of The Poly A Tract mRNA isolation systems (Promega). The cDNA was synthesized using a ZAP-cDNA Synthesis Kit (Stratagene) and fused to GAL4 transactivation domain (AD) in pAD-GAL4-2.1 phagemid vector. The library contained approximately 2×10^7 independent clones. The generation of the GAL4 activiation domain (AD) cDNA library in pAD-GAL4 (Stratagene) was done using the HybriZAP vectors Cloning System (Stratagene). The cloned vector was digested with *Eco*RI and *Pst*I, then ligated in pBD-Gal4 Cam vector to fuse to GAL4 DNA binding domain (BD). The entire coding region of *TBZF* was digested with *Eco*RI and filled in Klenow fragment to make blunt

end. After blunting, the DNA fragment was ligated to pGBT9 vector at *Sma*I site. The cDNA clones were rescued by in vivo excision using a helper phage (ExAssist, Stratagene), and the construct was confirmed by DNA sequencing.

Yeast transformation and two-hybrid assays

Yeast strain Y190 was transformed with pBD-TBZF and library plasmids by the polyethylene glycol/lithium acetate method (Elble, 1992). Transformants (1×10^6) harboring the bait and pray plasmids were screened on plates containing 20 mM 3-aminotriazole (3-AT) in synthetic dextrose (SD) medium without Trp, Leu and His. Interaction assays were performed on plates containing 0, 25, 50 or 75 mM 3-AT in SD medium without Trp, Leu and His. Empty vector pAD-GAL4-2.1 was used as the prey for control interaction with TBZF. Liquid β-galactosidase activity assay was according to the manufacturer's instructions (Promega). For β-galactosidase activity assay, Y190 was used and transformants were grown overnight in SD selection medium, diluted 1 to 5 in YPDA medium and grown for an additional 3 to 5 h at 30°C. Independent three colonies were assayed for each constructs. β-galactosidase activity was assessed in a 5-bromo-4-chloro-3-indolyl β-D-galactoside filter-lifting assay. For quantitative assays, *o*-nitrophenyl β-D-galactopyranoside was used as a substrate and β-galactosidase activity (U) was calculated as U = 1000 x (OD_{420}) / [Time (min) x Vol (ml) x (OD_{600})].

RNA isolation and northern hybridization

Extraction of total RNA and RNA blot analysis were carried out as described in Chapter I.

Construction of GFP-fusion plasmids and fluorescence microscopy

GFP-fusion plasmids and fluorescence microscopy were carried out as described in Chapter II.

Results

Isolation of TBZF interaction factors by yeast-two hybrid system

In order to identify proteins which interact with TBZF, a cDNA library containing 1×10^7 independent clones was constructed from mRNAs isolated from tobacco seedlings, and screened using yeast two-hybrid system. Prior to screening, I confirmed that the bait construct, containing pBD-TBZF fusion protein, does not activate transcription of reporter genes by itself (data not shown). This plasmid, pBD-TBZF, was introduced into the yeast strain Y190, and the transformants were tested for activation of the *HIS3* selectable marker. A cDNA expression library constructed from the mRNA of tobacco seedlings was introduced into the Y190 yeast strain containing pBD-TBZF. A total of 1.8×10^6 transformants was screened for their ability to grow on a medium without His. This initial screening identified 135 colonies, which were subsequently tested for activation of the *LacZ* gene. 63 colonies were positive in activation of both *HIS3* and *LacZ*. Plasmid DNAs were prepared from these colonies and retransferred into the Y190 strain to confirm whether the activation is indeed due to the presence of the fusion protein. I observed that 16 plasmids were able to activate the LacZ gene only in the presence of pBD-TBZF (Table 3). They were designated NtTIP (*N.tabacum* TBZF interacting protein) with appropriate serial numbered.

Interaction of TBZF and candidate proteins

NtTIPs were fused to the activation domain of GAL4 using the GAL4-pAD vector, and introduced into the yeast strain Y190 containing the binding domain plasmid pBD-TBZF. The colonies that grew on a medium lacking Leu and Trp were examined for β-galactosidase activity. The results showed that pAD-NtTIP1, pAD-NtTIP2, pAD-NtTIP8, and pAD-NtTIP15 were able to activate the LacZ gene (Fig. 38A). Other pAD-NtTIPs were unable to activate the reporter gene. Yeast cells co-transformed with pBD-TBZF and pAD-NtTIP1 grew on a selection medium containing 75 mM 3-AT, an inhibitor of HIS3 product, while other decreasing NtTIPs did not allow yeast to grow under this concentration of 3-AT. The interactions of pBD-TBZF and pAD-NtTIP1 were also detected by the filter assay of β-galactosidase (Fig. 38B). The β-galactosidase activity assay of each combination showed a

strong interaction between pBD-TBZF and pAD-NtTIP1 (Fig. 38C).

Isolation of NtTIP1 cDNA

Since the *NtTIP1* clone selected by the two-hybrid screening was partial, the 5' region was further isolated by PCR using a vector primer and cDNA specific primer. A cDNA clone of 776 bp, containing the entire ORF, was generated by connecting the 5' region to the cDNA clone obtained from the two-hybrid screening. This was designated NtTIP1 (*N.tabacum* TBZF interacting protein1). The *NtTIP1* encodes a putative protein of 12.5 kDa (Fig. 39A). The cDNA sequence (*NtTIP1*) was highly homologous to a transcript of tomato induced by water deficit and during ripening (Iusem et al., 1993), to a gene encoding a water-stress-induced protein from *Solanum chacoense* (Silhavy et al., 1995) and to a transcript of *solanum tuberosum* induced by cold, ABA and various other stress treatments (Scheider et al., 1997). Figure 39B shows the phylogenic tree deduced four amino acid sequences of this family (ASR/CI21 family).

Nuclear localization

To determine the cellular localization of NtTIP1 protein, the *NtTIP1* cDNA was fused to *GFP* gene under the control by cauliflower mosaic virus (CaMV) 35S promoter. The construct was transiently transformed into onion epidermal cells. The fluorescent signals were observed exclusively in nuclei (Fig. 40).

Expression pattern of NtTIP1

Expression pattern of *NtTIP1* and *TBZF* were determined by northern hybridization in leaf, root and flower tissues. Developmental change in *TBZF* and *NtTIP1* transcripts was also analyzed in flower development. Accumulation patterns of was essentially the same between the two, being high in flower and senescing leaves (Fig. 41A). Monitoring of *NtTIP1* transcripts during flower development and flower organs by northern analysis revealed the same results in both *TBZF* and *NtTIP1* (Fig. 41B and C).

The response of *NtTIP1* gene to abiotic stresses was examined by northern hybridization

analysis. The *TBZF* transcripts were upregulated by cold and ABA treatment for 6h (Fig. 42A). Drying for 1h induced *TBZF* expression but high salt and heat shock had no effect. *NtTIP1* transcripts were strongly induced by cold, ABA and drought, whereas other treatments were without obvious influence (Fig. 42A). Investigation of effects of phytohormones demonstrated *TBZF* and *NtTIP1* transcripts to be induced by ethylene, IAA and JA, but not by BA (Fig. 42B). It was concluded that transcripts for TBZF and NtTIP1 were synchronously accumulated biotic and abiotic stresses.

Discussion

In this chapter, I described that a gene encoding a heterodimerisation partner for TBZF was isolated by screening a tobacco library using *TBZF* as a probe. On the basis of deduced amino acid sequences, the *NtTIP1* gene was found to be a member of into the CI21 subfamily (van Berkel et al., 1994). They belong to the ASR/CI21 family, of which transcripts are induced by external stimuli and during fruit ripening (Schneider et al., 1997) (Fig. 39). For example, *ci21A* and *ci21B* of potato were shown to be induced upon cold-, ABA- and osmotic-stress (van Berkel et al., 1994; Schneider et al., 1997). Tomato *ASR1* transcripts were induced by water deficit and during ripening (Iusem et al., 1993; Rossi and Iusem, 1994). Expression of *ASR/CI21* gene family was also reported to be regulated during developmental process (Gillapsy et al., 1993; Meekswager, 1993; Thomas, 1993; Iusem et al., 1993). However, their physiological function has so far been not clear at all.

As to bZIP, there is only one report describing isolation of bZIP interacting proteins, BZI-1 (a TBZ-type transcription factors) (Strathmann et al., 2001). All of them were bZIP-type protein and shown to form heterodimers (Landschultz et al., 1988; Vinson et al., 1993). Northern analysis revealed that BZI-2, BZI-3/TBZF and BZI-4 were predominantly expressed in flowers and, to a lesser extent, in the vegetative parts of the plant (Strathmann et al., 2001). However, nothing is known how bZIP form transcription complexes to initiate gene expression.

In the present study, I found a clear interaction between NtTIP1 and TBZF. Both localized in nucleus, and both transcripts were synchronously induced during senescence and flower development. These observations strongly suggest that they form a complex in vivo, possibly functioning in regulation of relevant genes to senescence. Although the exact interacting mode between the two is not clear yet, it is conceivable that this transcription complex plays a central role in switch-on of many genes simultaneously. To my knowledge, this is the first report that bZIP interacts with a non-bZIP protein, and will contribute to advance our understandings not only in protein-protein interaction, but also in the mode of global regulation of genes by, for example, regulator system.

Table 3 Clones isolated by yeast two-hybrid system.

Clone No.	Candidate interacting proteins	Species	Subcellular location	Organ	Acces. No.
1	ci21A (cold regulated gene ci21A)	S. tuber.	nuclear	tuber, leaf	U76610
2	SKP1-like protein mRNA	N. clevelandii			AF070967
3, 4	Metallothionein-like protein Type 2	N. glutinosa			Q40396
5, 14	NelF-5A2 for initation factor 5A2	N. plumb.		leaf, stem, flower	X63542
6	Calcium-dependent protein OsCDPK7	O. sativa			BAB16888
7	Photosystem II 10kd polypeptide (PII 10)	N. tabacum	chloroplast		Q40519
8	Peptidyl-prolyl cis-trans isomeras	A. thaliana	cytoplast	flower, YL, root	AAD20122
9, 10	Photosystem I psaH precursor	N. sylvestris			T16958
11, 12	Glyceraldehyde-3-phosphate dehydrogenase	N. tabacum	cytoplasm	root, epi, xylem	CAB39974
13	Thioredoxin H-type 1 (TRX-H1)	N. tabacum			P29449
14	NelF-5A2 for initation factor 5A2	N. plumb.		leaf, stem, flower	X63542
15	Chlorophyll a/b-binding protein	N. sylvestris	chloroplast		BAA25392
16	Tubulin beta-2/beta-3 chain	A. thaliana		root,stem, flower	P29512

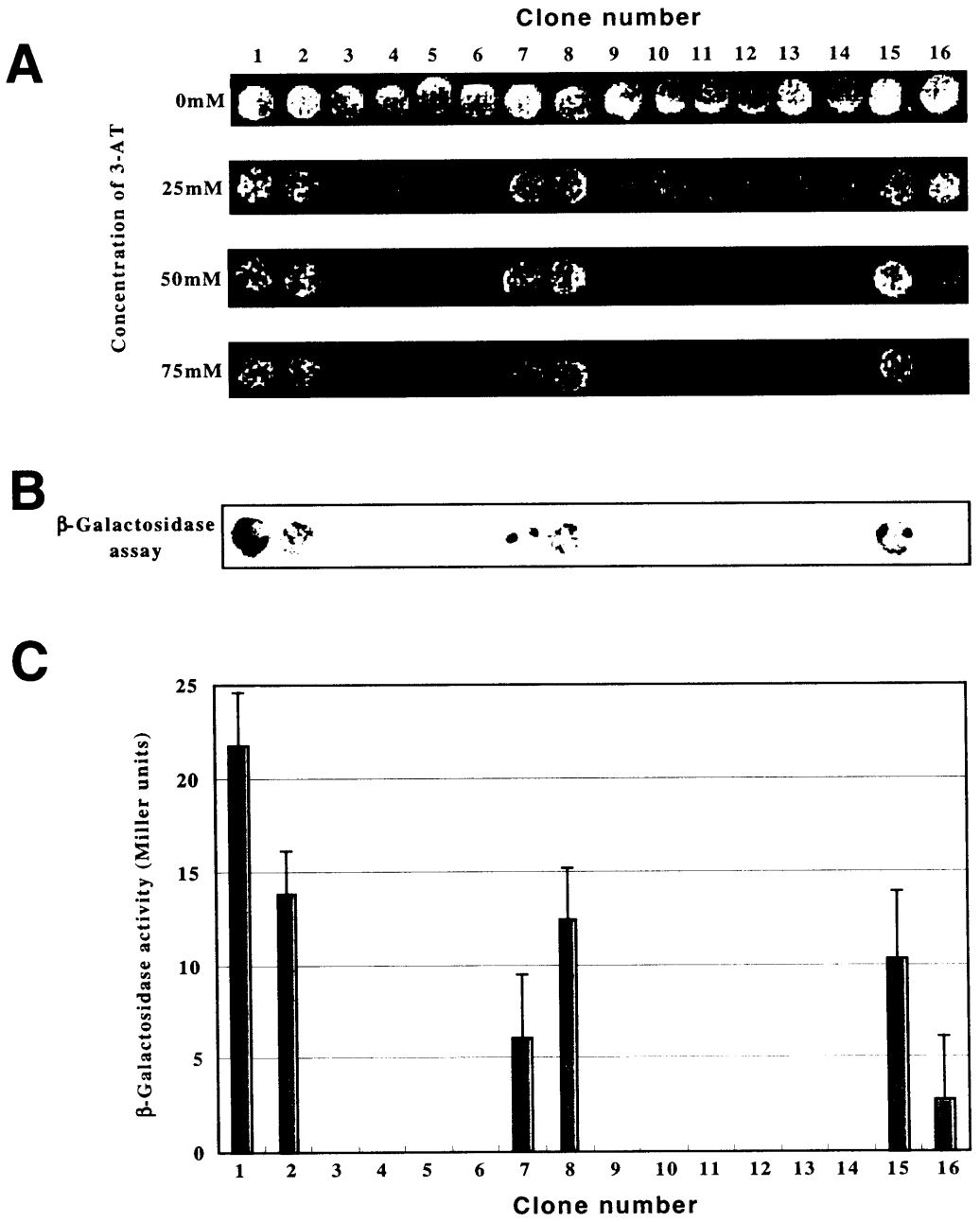


Fig. 38 Interaction between TBZF and cadidate proteins identified by the yeast two-hybrid system. (**A**) Yeast two-hybrid assay. pBD-TBZF and pAD-cadidate plasmids were introduced into Y190 yeast cells and their growth on the SD medium (-Leu/Trp/His) containing 3-AT (0, 25, 50 or 75 mM) was monitored. The empty vector pAD-GAL4-2.1 was used as prey for the control interaction with TBZF. Numbers correspond to clones listed in Table 1. (**B**) β-galactosidase assays. Colonies grown on SD medium (-LTH, 0 mM 3-AT) were used for filter-lift assay. (**C**) Measurement of β-galactosidase activities. Three individual transformants were used to measure the β-galactosidase activity as described in "Materials and Methods". Vertical bars represent standard deviations.

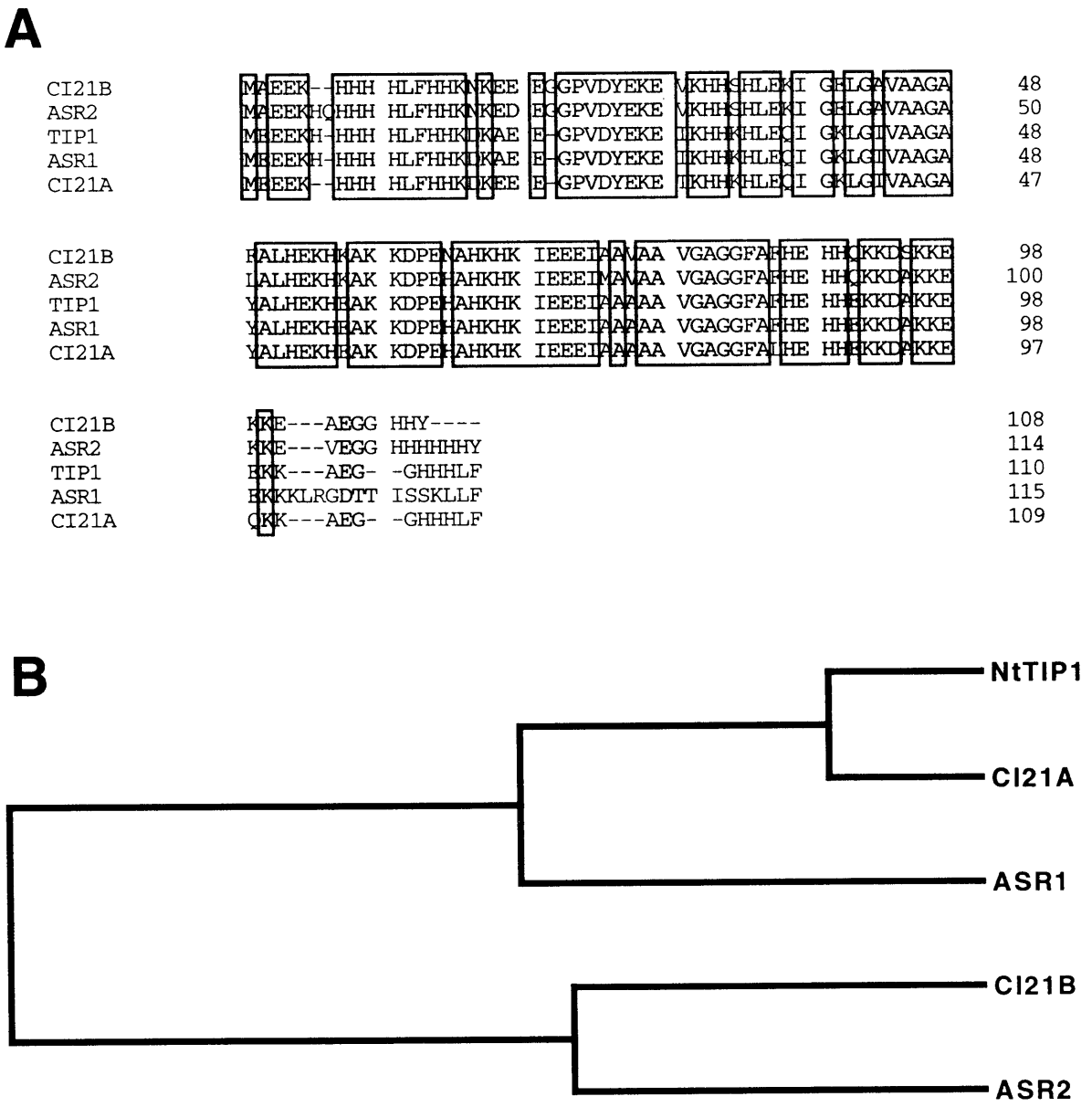


Fig. 39 (**A**) Amino acid sequence alignment of NtTIP1 and other CI21/ASR familys. (**B**) A phylogenic tree among CI21/ASR family proteins. Comparison among the amino acid sequences predicted from the CI21 cDNA (CI21A), the coding sequence of gene *ci21B* (CI21B), the ripening-related and water-deficiency-induced transcript ASR1 from tomato (Rossi and Iusem, 1994), the coding sequence of tomato gene asr2 (ASR2, Rossi and Iusem, 1994), and the senescence-related and stress-induced transcript TIP1 from tobacco (in this thesis).

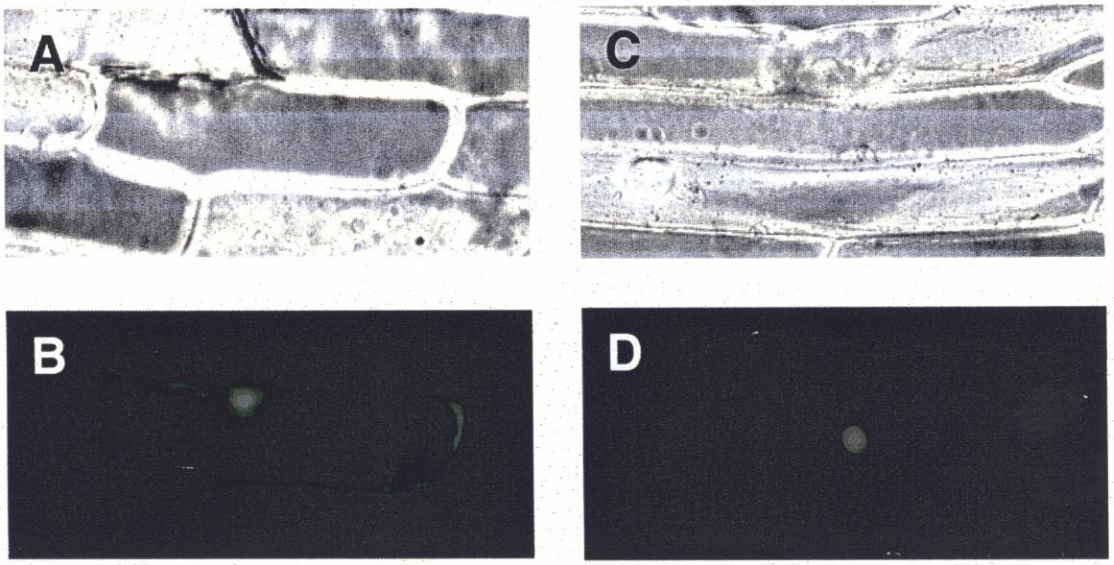


Fig. 40 Nuclear localization of NtTIP1 in onion epidermal cells. Onion bulbs were bombarded with gold particles coated with pGFP2 (A, B) and pNtTIP1::GFP plasmids (C, D) . Proteins were transiently expressed and individual cells were observed by epifluorescence (A and C) and corresponding differential interference contrast imaging (B and D).

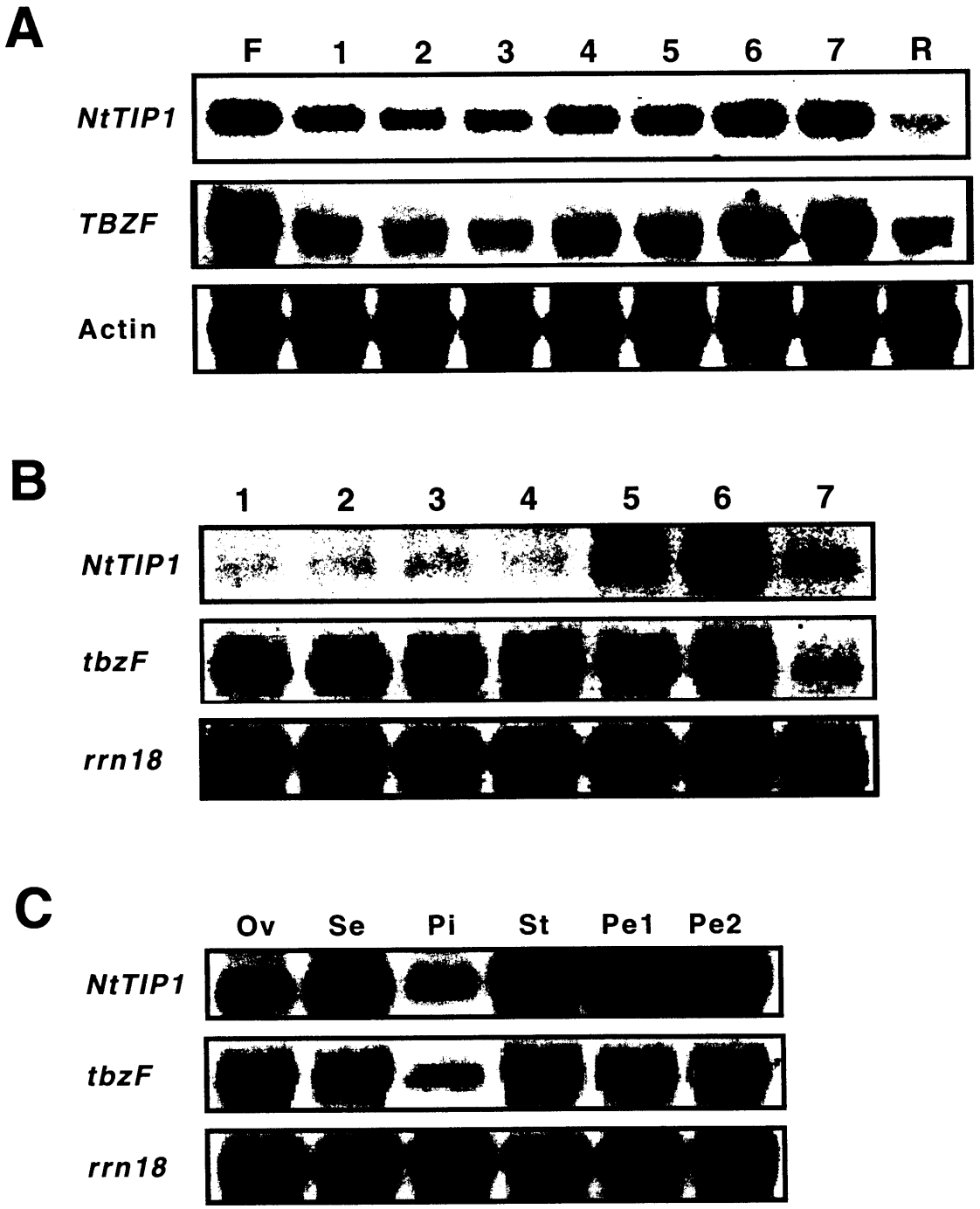


Fig. 41 Tissue-specific expression of *NtTIP1* and *TBZF* genes. (**A**) Total RNAs were also extracted from the same tissues and the abundance of *NtTIP1* and *TBZF* transcripts were estimated by northern hybridization using the respective ORF or 3'-UTR regions. The same filter was subsequently hybridized with the tobacco *actin*- or *rrn18*-cDNA (*actin*, Thangavelu et al., 1993; *rrn18*, Ganal and Hemleben, 1986) to ensure equal sample loading. floral parts (F), root (R), leaves are numbered from the base to the tip. (**B**) Abundance of *NtTIP1* and *tbzF*-transcripts at flower developmental stages. Numbers is flower with completely opened petals (stage 1). (**C**) Floral parts expressed in *NtTIP1* and *TBZF* genes. ovule (O), sepal (Se), pistil (Pi), pink part of petal (Pe1) and lower white part of petal (Pe2).

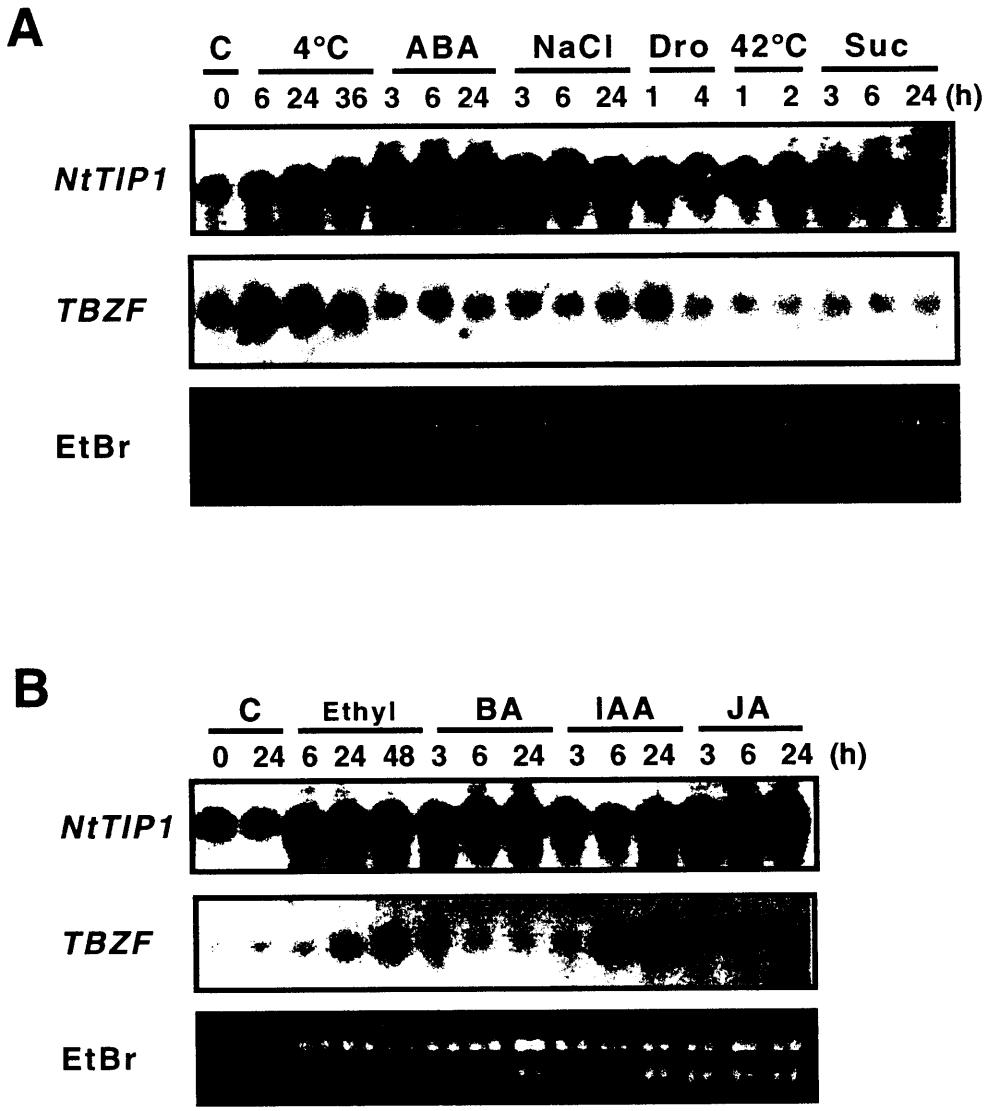


Fig. 42 Response of *NtTIP1* and *TBZF* genes in tobacco plants subjected to several abiotic stresses and hormonal treatments. Tobacco seedlings were subjected to abiotic stresses and hormone treatment for indicated time intervals as described in Materials and Methods. (**A**) control (Cont), 4°C, abscisic acid (ABA), 0.2 M NaCl-treated (NaCl), air-dried (Dro) and heat shocked (42°C). (**B**) ethylene-treated (Ethyl), benzyladenine (BA), indole acetic acid (IAA) and jasmonic acid (JA)

Concluding remarks

During senescence nutrients are mobilized from senescing leaves to developing parts of the plant, such as growing leaves, developing flowers, seeds or storage organs after breakdown of cellular structures (Noh and Amasino, 1999). As an SAG, *Ntdin* transcripts were not only accumulated during senescence, but also by sulfate and nitrate treatment. Further analysis showed that, in senescing leaves, Ntdin induced the Moco activity to molybdoenzymes which are involved in recycling system of nutrients. Indeed, transgenic plants expressing antisense *Ntdin* showed lower activity of molybdoenzymes such as NR, XDH and AO, incomparison with those of wild-type. These results suggested that Ntdin functions in sulfate and nitrate recycling system, which is activited during proceeding of senescence. TBZF and TBZ17 are possibly involved in controlling transcription of SAGs functioning in guard cells of senescing leaves. Recently, TBZF was shown to form a heterodimer with BZI-1, another bZIP protein in tobacco plants. However, there is no report on TBZF interacting non-bZIP protein. In this study, I found a TBZF interacting protein NtTIP1, which is a member of the CI21 subfamily. Although CI21s were shown to respond to environmental stimuli, such as drought and cold, their physiological function has so far been not clear at all. TBZF and NtTIP1 are localized in nucleus, and both transcripts were synchronously induced during senescence. These observations strongly suggest that they form a complex in vivo, possibly functioning in regulation of relevant genes during senescence. At least four CI21 related genes have been reported, and current finding points their function to participate in transcriptional activation of SAGs through interacting with bZIPs.

Taken together, I propose the following working model (Fig. 43). Ntdin may control the activity of nitrate reduction enzyme through formation of Moco of sulfurylated form. Since, *Ntdin* transcripts are induced by darkness, sulfate, nitrate and during natural senescence, *Ntdin* is possibly involved in intracellular recycling of sulfate and nitrate translocation, through the promotion of specific enzymatic activity such as NR. In additions to metabolic enzymes that require the Moco, several enzymes in the biosynthetic pathway of ABA and auxin, that are involved in senescence processes, also require this cofactor (Fig.

43A). Senescence signal directly activates *tbzF* and *NtTIP1*, and resulting proteins form a complex, which activates senescence-associated genes (Fig. 43B). This complex may recognize the G-box element (ACGTG), which are found in many SAGs including Ntdin (Fig. 43C).

Overall, the present study suggests (1) the presence of a coordinated system, in which activation/ inactivation of SAGs is regulated by bZIP complex, and (2) one of such SAGs to be involved in recycling system of accumulated materials.

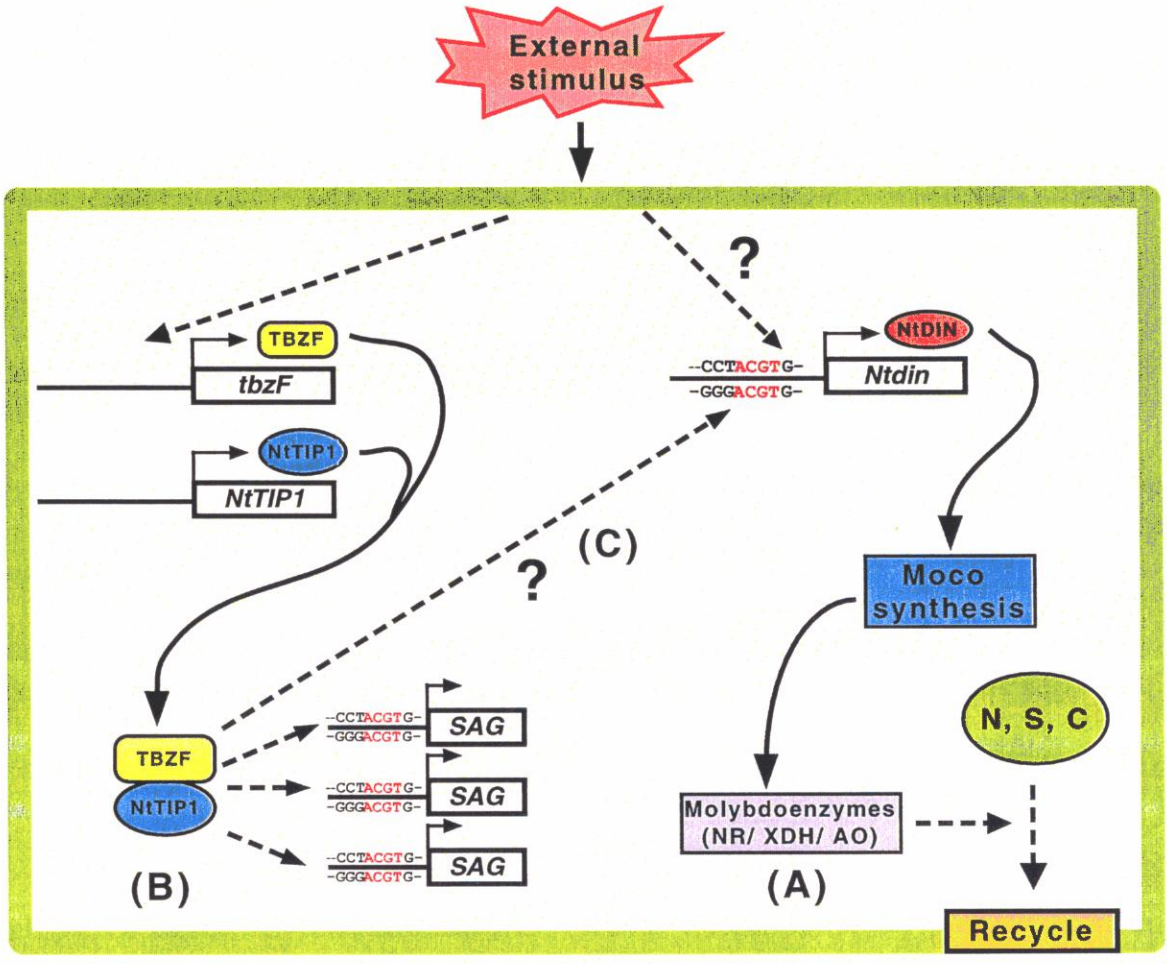


Fig. 43 Working hypothesis for tbzF, NtTIP1 and Ntdin.

References

Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997) Role of arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* 9: 1859-1868.

Abeles, F.B., Dunn, L.J., Morgens, P., Callaha, A., Dinterman, R.E., and Schmidt, J. (1988). Induction of 33-kD and 60-kD peroxidases during ethylene-induced senescence of cucumber cotyledons. *Plant Physiol.* 87; 609-615.

Aguan, K., Sugawara, K., Suzuki, N., and Kusano T. (1993) Low-temperature-dependent expression of a rice gene encoding a protein with a leucine-zipper motif. *Mol. Gen. Genet.* 240: 1-8.

Altschul S.F., Gish W., Miller W., Myers E.W., and Lipman D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.

Armstrong, G.A., Weisshaar, B., and Hahlbrock, K. (1992) Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. *Plant Cell* 4: 525-537.

Assmann, S.M. (1993) Signal transduction in guard cells. *Annu. Rev. Cell Biol.* 9: 345-375.

Azumi Y., and Watanabe A. (1991) Evidence for a senescence-associated gene induced by darkness. *Plant Physiol.* 95: 577-583.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994) The 5'-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.* 24: 701-13.

Bate, N.J., Rothstein, S.J., and Thompson, J.E. (1991) Expression of nuclear and chloroplast photosynthesis-specific genes during leaf senescence. *J. of Exp. Bot.* 42: 801-811.

Bate, N., and Twell, D. (1998) Functional architecture of a late pollen promoter: pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Mol. Biol.* 37: 859-869.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 92: 8675-8679.

Berberich, T., Sano, H., and Kusano, T. (1999) Involvement of a MAP kinase, ZmMPK5, in senescence and recovery from low-temperature stress in maize. *Mol. Gen. Genet.* 262: 534-542.

Biedenkapp, H., Borgmeyer, U., Sippel, A.E., and Klempnauer, K.H. (1988) myb oncogene encodes a sequence-specific DNA-binding activity. *Nature* 335: 835-7.

Blank, A., and McKeon, T.A. (1991) Expression of three RNase activities during natural and dark-induced senescence of wheat leaves. *Plant Physiol.* 97: 1409-1413.

Bleecker, A.B. (1998) The evolutionary basis of leaf senescence: method to the madness? *Curr. Opin. Plant Biol.* 1: 73-8.

Bowen, I.D. (1993) Apoptosis or programmed cell death? *Cell Biol. Int.* 17: 365-80.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 7: 248-254.

Buchanan-Wollaston, Y. (1997) The molecular biology of leaf senescence. *J. Exp. Bot.* 48: 181-199.

Buchanan-Wollaston, V., and Ainsworth, C. (1997) Leaf senescence in Brassica napus: cloning of senescence related genes by subtractive hybridisation. *Plant Mol. Biol.* 33: 821-34.

Busk, P.K., and Pages, M. (1998) Regulation of abscisic acid-induced transcription. *Plant Mol. Biol.* 37: 425-435.

Callard, D., Axelos, M., and Mazzolini, L. (1996) Novel molecular markers for late phases of the growth cycle of Arabidopsis thaliana cell-suspension cultures are expressed during organ senescence. *Plant Physiol.* 112: 705-15.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89: 1133-1144.

Chomczynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159.

Chuang, C.F., Running, M.P., Williams, R.W., and Meyerowitz, E.M. (1999) The PERIANTHIA gene encodes a bZIP protein involved in the determination of floral organ number in Arabidopsis thaliana. *Genes Dev.* 13: 334-44.

Chung, K.M,, Song, O.K., and Jang, S.K. (1997) Hepatitis C virus nonstructural protein 5A contains potential transcriptional activator domains. *Mol. Cell* 7: 661-667.

Chung, K.M., Lee, J., Kim, J.E., Song, O.K., Cho, S., Lim, J., Seedorf, M., Hahm, B., and Jang, S.K. (2000) Nonstructural protein 5A of hepatitis C virus inhibits the function of karyopherin β3. *J. Virol.* 74: 5233-5241.

Church, G.M., and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. U S A* 81:

1991-5.

Clausen, S., and Apel, K. (1991) Seasonal changes in the concentration of the major storage protein and its mRNA in xylem ray cells of poplar trees. *Plant Mol. Biol.* 17: 669-78.

Creelman, R.A., Tierney, M.L., and Mullet, J.E. (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. USA* 89: 4938-4941.

Crowell, D.N., John, M.E., Russell, D., and Amasino, R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol. Biol.* 18: 459-66.

Crafts-Brandner, S.J., Klein, R.R., Klein, P., Holzer, R., and Feller, U. (1996) Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of the Clp protease subunits during senescence of Phaseolus vulgaris (L.) leaves. *Planta* 200: 312-8.

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., and Granot, D. (1999) Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. *Plant Cell* 11: 1253-66.

Dixon, C.D., Cutt, J.R., and Klessig, D.F. (1995) *In situ* hybridization for the detection of RNA in plant tissues. *In* P Maliga, DF Klessig, AR Cashmore, W Gruissem, JE Varner, eds, Methods in Plant Molecular Biology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 111-137.

Dooley, T.P., Nair, S.K., Garcia, IV R.E., and Courtney, B.C. (1995) Mouse rhodanese gene (*Tst*): cDNA cloning, sequencing, and recombinant protein expression. *Biochem. Biophys. Res. Comm.* 216: 1101-1109.

Drake, R., John, I., Farrell, A., Cooper, W., Schuch, W., and Grierson, D. (1996) Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. Plant Mol. Biol. 30: 755-67.

Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. *Biotechniques* 13: 18-20.

Eyal, Y., Curie, C., and McCormick, S. (1995) Pollen specificity elements reside in 30 bp of the proximal promoters of two pollen-expressed genes. *Plant Cell* 7: 373-84.

Feller, U., and Fisher, A. (1994) Nitrogen metabolism in senescing leaves. *Crit. Rev. Plant Sci.* 13: 241-273.

Fukazawa, J., Sakai, T., Ishida, S., Yamaguchi, I., Kamiya, Y., and Takahashi, Y. (2000) Repression of shoot growth, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. *Plant Cell* 12: 901-15.

Gan, S., and Amasino, R.M. (1997) Making sense of senescence: molecular genetic regulation and manipulation of leaf senescence. *Plant Physiol.* 113: 313-319.

Ganal, M., and Hemleben, V. (1986) Comparison of the ribosomal RNA genes in four closely related *Cucurbitaceae*. *Plant Syst. Evol.* 154: 63-77.

Garbarino, J.E., Oosumi, T., and Belknap, W.R. (1995) Isolation of a polyubiquitin promoter and its expression in transgenic potato plants. *Plant Physiol.* 109:1371-1378.

Gay, A.P., and Thomas, H. (1995) Leaf development in Lolium temulentum L: photosynthesis in relation to growrh and senescence. *New Phytol.* 130: 159-168.

Gillapsy, G., Bendavid, H., and Gruissem, W. (1993) Fruits - a developmental perspective. *Plant Cell* 5: 1439-1452.

Gilmour, S.J., Artus, N.N., and Thomashow, M.F. (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol. Biol.* 18:13-21.

Grbic, V., and Bleecker, A.B. (1995) Ethylene regulates the timing of leaf senescence in Arabidopsis. *Plant J.* 8: 595-602.

Graham, I.A., Leaver, C.J., and Smith, S.M. (1992) Induction of malate synthase gene expression in senescent and detached organs of cucumber. *Plant Cell* 4: 349-357.

Hamilton, D.A., Schwarz, Y.H., and Mascarenhas, J.P. (1998) A monocot pollen-specific promoter contains separable pollen-specific and quantitative elements. *Plant Mol. Biol.* 38: 663-669.

Hanfrey, C., Fife, M., and Buchanan-Wollaston, V. (1996) Leaf senescence in Brassica napus: expression of genes encoding pathogenesis-related proteins. *Plant Mol. Biol.* 30: 597-609.

Hara, K., Yagi, M., Kusano, T., and Sano, H. (2000) Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding. *Mol. Gen. Genet.* 263: 30-37.

Hensel, L.L., Grbic, V., Baumgarten, D.A., and Bleeker, A.B. (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. *Plant Cell* 5: 553-564.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* 27: 297-300.

Hoff, T., Schnorr, K.M., Meyer, C., and Caboche, M. (1995) Isolation of two Arabidopsis cDNAs involved in early steps of molybdenum cofactor biosynthesis by functional

complementation of *Escherichia coli* mutants. *J. Biol. Chem.* 270: 6100-6107.

Hsieh, H.M., Liu, W.K., and Huang, P.C. (1995) A novel stress-inducible metallothionein-like gene from rice. *Plant Mol. Biol.* 28: 381-9.

Hurst, H. (1994) Transcription factors. 1. bZIP proteins. *Protein Profiles* 1: 123-168.

Ito, K., Kusano, T., and Tsutsumi, K. (1999) Cold-inducible bZIP protein gene in radish root regulated by calcium- and cycloheximide-mediated signals. *Plant Science* 142: 57-66.

Iusem, N.D., Bartholomew, D.M., Hitz, W.D., and Scolnik, P.A. (1993) Tomato (*Lycopersicon esculentum*) transcript induced by water deficit and ripening. *Plant Physiol.* 102:1353-1354.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion systerm. *Plant Mol. Biol.* 30: 679-684.

Jackson, D.P. (1991) In situ hybridisation in plants. In *Molecular Plant Pathology: A Practical Approach* (Bowles, D.J., Gurr, S.J. and McPhereson, M., eds). Oxford: IRL Press, pp. 163-174.

Jepson, I., Lay, V.J., Holt, D.C., Bright, S.W., and Greenland, A.J. (1994) Cloning and characterization of maize herbicide safener-induced cDNAs encoding subunits of glutathione S-transferase isoforms I, II and IV. *Plant Mol. Biol.* 26: 1855-66.

John, I., Hackett, R., Cooper, W., Drake, R., Farrell. A., and Grierson, D. (1997) Cloning and characterization of tomato leaf senescence-related cDNAs. *Plant Mol. Biol.* 33: 641-651.

Kamada, I., Yamauchi, S., Youssefian, S., and Sano, H. (1992) Transgenic tobacco plants expressing *rgp1*, a gene encoding a ras-related GTP-binding protein from rice, show distinct morphological characteristics. *Plant J.* 2: 799-807.

King, G.A., Davies, K.M., Stewart, R.J., and Borst, W.M. (1995) Similarities in gene expression during the postharvest-induced senescence of spears and the natural foliar senescence of asparagus. *Plant Physiol.* 108: 125-128.

Kisker, C., Schindelin, H., and Rees, D.C. (1997) Molybdenum-cofactor-containing enzymes: structure and mechanism. *Annu. Rev. Biochem.* 66: 233-267.

Kleber-Janke, T., and Krupinska, K. (1997) Isolation of cDNA clones for genes showing enhanced expression in barley leaves during dark-induced senescence as well as during senescence under field conditions. Planta 203: 332-340.

Koshiba, T., Saito, E., Ono, N., Yamamoto, N., Sato, M. (1996) Purification and properties of flavin- and molybdenum-containing aldehyde oxidase from coleoptiles of maize. *Plant Physiol.* 110: 781-789.

Kopka, J., Provart, N.J., and Mueller-Röber, B. (1997) Potato guard cells respond to drying soil by a complex change in the expression of genes regulated to carbon metabolism and turgor regulation. *Plant J.* 11: 871-882.

Kosugi, S., and Ohashi, Y. (2000) Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. *Nucleic Acids Res.* 28: 960-967.

Kouchi, H., and Hata, S. (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol. Gen. Genet.* 238: 106-119.

Kurkela, S., and Frank, M. (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol. Biol.* 15: 137-144.

Kusano, T., Berberich, T., Harada, M., Suzuki, N., and Sugawara, K. (1995) A maize DNA binding factor with bZIP motif is induced by low temperature. *Mol. Gen. Genet.* 248: 1-8.

Kusano, T., Sugawara, K., Harada, M., and Berberich, T. (1998) Molecular cloning and partial characterization of a tobacco cDNA encoding a small bZIP protein. *Biochim. Biophys. Acta.* 1395: 171-175.

Kwak, J.M., Kim, S., Hong, S.W., and Nam, H.G. (1997) Evaluation of 515 expressed sequence tags obtained from guard cells of *Brassica campestris*. *Planta* 202: 9-17.

Kyhse-Andersen, J. (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* 10: 203-209.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L, (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759-1763.

Leung, J., Merlot, S., and Giraudat, J. (1997) The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* 9: 759-771.

Li, J., Wang, X-Q., Watson, M.B., and Assmann, S.M. (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* 287: 300-303.

Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: Pigment of photosynthetic biomembranes. *Methods Enzymol.* 148: 350-382.

Lipman, D.J., and Pearson, W.R. (1985) Rapid and sensitive protein similarity searches. *Science* 227: 1435-1441.

Liu, Y.G., Mitsukawa, N., and Whittier, R.F. (1993) Rapid sequencing of unpurified PCR products by thermal asymmetric PCR cycle sequencing using unlabeled sequencing primers. *Nucleic Acids Res.* 21: 3333-3334.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) The transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* 10: 1391-1406.

Lohman, K.N., Gan, S., John, M.C., and Amasino, R.M. (1994) Molecular analysis of natural leaf senescence in Arabidopsis thaliana. *Physiol. Plant* 92: 322-328.

Luo, G-X., and Horowitz, P.M. (1994) The sulfurtransferase activity and structure of rhodanese are affected by site-directed replacement of Arg-186 and Lys-249. *J. Biol. Chem.* 269: 8220-8225.

Mandel, T., Fleming, A.J., Krahenbuhl, R., and Kuhlemeier, C. (1995) Definition of constitutive gene expression in plants: the translation initiation factor 4A gene as a model. *Plant Mol. Biol.* 29: 995-1004.

Marcotte, W.R. Jr., Russell, S.H., and Quatrano, R.S. (1989) Abscisic acid-responsive sequences from the em gene of wheat. *Plant Cell* 1: 969-976.

Matile, P. (1992) Chloroplast senescence. *In Crop Photosynthesis: Spatial and Temporal Determinants* (Bake, N. and Thomas, H., eds). Amsterdam: Elsevier, pp. 413-440.

Matile, P., Hoertensteiner, S., Thomas, H., and Kraeutler, B. (1996) Chlorophyll breakdown in senescent leaves. *Plant Physiol.* 112: 1403-1409.

Martínez-García, J.F., Moyano, E., Alcocer, M.J.C., and Martin, C. (1998) Two bZIP proteins from *Antirrhinum* flowers preferentially bind a hybrid C-box/G-box motif and help to define a new sub-family of bZIP transcription factors. *Plant J.* 13: 489-505.

Matsuo, N., Minami, M., Maeda, T., and Hiratsuka, K. (2001) Dual luciferase assay for monitoring transient gene expression in higher plants. *Plant Biotech.* 18: 71-75.

Medina, J., Catalå, R., and Salinas, J. (2001) Developmental and stress regulation of RCI2A and RCI2B, two cold-inducible genes of arabidopsis encoding highly conserved hydrophobic proteins. *Plant Physiol.* 125: 1655-66.

Meekswager, D.R. (1993) Gene expression in the early floral meristem. *Plant Cell* 5: 1167-1174.

Mendel, R.R., and Müller, A.J. (1976) A common genetic determinant of xanthine

dehydrogenase and nitrate reductase in *Nicotiana tabacum*. *Biochem. Physiol. Pflanzen.* 170: S. 538-541.

Mendel, R.R., Alikulof, Z.A., Lvov, N.P., and Müller, A.J. (1981) Presence of the molybdenum-cofactor in nitrate reductase-deficient mutant cell lines of *Nicotiana tabacum*. *Mol. Gen. Genet.* 181: 395-399.

Mendel, R.R., Kirk, D.W., and Wray, J.L. (1985) Assay of molybdenum cofactor of barley. *Phytochemistry* 24: 1631-1634.

Mendel, R.R. (1997) Molybdenum cofactor of higher plants: biosynthesis and molecular biology. *Planta* 203: 399-405.

Menke, U., Renault, N., and Müeller-Röber, B. (2000) StGCPRP, a potato gene strongly expressed in stomatal guard cell, defines a novel type of repetitive proline-rich proteins. *Plant Physiol.* 122: 677-686.

Meshi, T., and Iwabuchi, M. (1995) Plant transcription factors. *Plant Cell Physiol.* 36: 1405-1420.

Mikami, K., Katsura, M., Ito, T., Okada, K., Shimura, Y., and Iwabuchi, M. (1995) Developmental and tissue-specific regulation of the gene for the wheat basic/leucine zipper protein HBP-1a(17) in transgenic Arabidopsis plants. *Mol. Gen. Genet.* 248: 573-82.

Mitsuda, N., Takeyasu, K., and Sato, M.H. (2001) Pollen-specific regulation of vacuolar H+-PPase expression by multiple *cis*-acting elements. *Plant Mol. Biol.* 46: 185-92.

Moon, Y.H., Kang, H.G., Jung, J.Y., Jeon, J.S., Sung, S.K., and An, G. (1999) Determination of the motif responsible for interaction between the rice APETALA1/AGAMOUS-LIKE9 family proteins using a yeast two-hybrid system. *Plant Physiol.* 120: 1193-1204.

Moriau, L., Bogaerts, P., Jonniaux, J.L., and Boutry, M. (1993) Identification and characterization of a second plasma membrane H(+)-ATPase gene subfamily in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* 21: 955-963.

Morris, K., Thomas, H., and Rogers, L. (1996) Endopeptidases during the developmental and senescence of Lolium temulentum leaves. *Phytochemistry* 41: 377-384.

Müller-Röber, B., La Cognata, U., Sonnewald, U., and Willmitzer, L. (1994) A truncated version of an ADP-glucose pyrophosphorylase promoter from potato specifies guard cell-selective expression in transgenic plants. *Plant Cell* 6: 601-661.

Müller-Röber, B., Ellenberg, J., Provart, N., Willmitzer, L., Busch, H., Becker, D., Dietrich, P., Hoth, S., and Hedrich, R, (1995) Cloning and electrophysiological analysis of KST1, an inward rectifying K+ channel expressed in potato guard cells. *EMBO J.* 14: 2409-2416.

Müller-Röber, B., Ehrhardt, T., and Plesch, G. (1998) Molecular features of stomatal guard cells. *J. Exp. Bot.* 49: 293-304.

Murray, M.G., and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.

Murre, C., McCaw, P.S., and Baltimore, D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56: 777-83.

Nagahara, N., Okazaki, T., and Nishino, T. (1995) Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese. *J. Biol. Chem.* 270: 16230-16235.

Nagahara, N., and Nishino, T. (1996) Role of amino acid residues in the active site of rat liver mercaptopyruvate sulfurtransferase. cDNA cloning, overexpression, and site-directed mutagenesis. *J. Biol. Chem.* 271: 27395-27401.

Nagata, T., Okada, K., Takebe, I., and Matsui, C. (1981) Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). *Mol. Gen. Genet.* 184: 161-165.

Nagy, F., Kay, S.A., and Chua, N.H. (1988) Analysis of gene expression in transgenic plants. In *Plant Molecular Biology Manual* (Gelvin SB and Schilperoort RA eds) Dordrecht. Kluwer Academic Publishers, B4: pp1-29.

Nakagoshi, H., Nagase, T., Kanei-Ishii, C., Ueno, Y., and Ishii, S. (1990) Binding of the c-myb proto-oncogene product to the simian virus 40 enhancer stimulates transcription. *J. Biol. Chem.* 265: 3479-3483.

Nakamura, R.L., McKendree, W.L. Jr., Hirsch, R.E., Sedbrook, J.C., Gaber, R.F., and Sussman, M.R. (1995) Expression of an Arabidopsis potassium channel gene in guard cells. *Plant Physiol.* 109: 371-374.

Nakano, Y., Steward, N., Sekine, M., Kusano, T., and Sano, H. (2000) A tobacco NtMet1 cDNA encoding a DNA methyltransferase: Molecular characterization and abnormal phenotypes of transgenic tobacco plants. *Plant Cell Physiol.* 41: 448-457.

Nagy, F., Kay, S.A., and Chua, N.H. (1988) Analysis of gene expression in transgenic plants. In *Plant Molecular Biology Manual* (Gelvin, S.B. and Schilperoort, R.A. eds) Dordrecht. Kluwer Academic Publishers, B4: pp1-29.

Nam, H.G. (1997) The molecular genetic analysis of leaf senescence. *Curr. Opin. Biotech.* 8: 200-207.

Noh, Y.S., and Amasino, R.M. (1999) Identification of a promoter region responsible for the

senescence-specific expression of SAG12. *Plant Mol. Biol.* 41: 181-194.

Noodén, L.D., and Leopold, A.C. (1978) Phytohormones and the endogenous regulation of senescence and abscission. In: Letham DS, Goodwin PB, Higgins TJV, eds. *Phytohormones and related compounds: a comprehensive treatise,* Vol. II. Amsterdam: Elsevier/North-Holland Biomedical Press, 329-369.

Noodén, L.D. (1980) Senescence in the whole plant. In: Thimann KV, ed. Senescence in plants. Boca Raton, FL: CRC Press, 219-258.

Noodén, L.D. (1988) The phenomenon of senescence and aging. In Senescence and Aging in Plants, L.D. Noodén and A.C. Leopold, eds (San Diego, CA: Academic Press) pp. 2-50.

Noodén, L.D., Guiamét, J.J., and John, I. (1997) Senescence mechanisms. *Physiol. Plant* 101: 746-753.

Nordin, K., Vahala, T., and Palva, E.T. (1993) Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* 21: 641-53.

Oh, S.A., Lee, S.Y., Chung, I.K., Lee, C.H., and Nam, H.G. (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. *Plant Mol. Biol.* 30: 739-754.

Oh, S.A., Park, J.H., Lee, G.I., Paek, K.H., Park, S.K., and Nam, H.G. (1997) Identification of three genetic loci controlling leaf senescence in Arabidopsis thaliana. Plant J. 12: 527-535.

Oyama, T., Shimura, Y., Okada, K. (1997) The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev. 11: 2983-2995.

Page, R.D.M. (1996) TREEVIEW: An application to display phylogenic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Pallini, R., Guazzi, G.C., Cannella, C., and Cacace, M.G. (1991) Cloning and sequence analysis of the human liver rhodanese: comparison with the bovine and chicken enzymes. *Biochem. Biophys. Res. Comm.* 180: 887-893.

Park, J.-H., Oh, S.A., Kim, Y.H., Woo, H.R., and Nam, H.G. (1998) Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. Plant Mol. Biol. 37: 445-454.

Parra, M.M., del. Pozo. O., Luna, R., Godoy, J.A., and Pintor-Toro, J.A. (1996) Structure of the dehydrin *tas14* gene of tomato and its developmental and environmental regulation in transgenic tobacco. *Plant Mol. Biol.* 32: 453-460.

Pla, M., Vilardell, J., Guiltinan, M.J., Marcotte, W.R., Niogret, M.F., Quatrano, R.S., and

Pages, M. (1993) The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab28*. *Plant Mol. Biol.* 21: 259-266.

Plesch, G., Kamann, E., and Müller-Röber, B. (2000) Cloning of regulatory sequences mediating guard-cell-specific gene expression. *Gene* 249: 83-89.

Plesch, G., Ehrhardt, T., and Mueller-Roeber, B. (2001) Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. *Plant J.* 28: 455-464.

Ploegman, J. H., Drent, G. Kalk, K. H., Heinrikson, R. L., Keim, P., Weng, L., and Russell, J. (1978) The covalent and tertiary structure of bovine liver rhodanese. *Nature* 273: 124-129.

Pysh, L.D., Aukerman, M.J., and Schmidt, R.J. (1993) OHP1: a maize basic domain/leucine zipper protein that interacts with opaque2. *Plant Cell* 5: 227-36.

Rajagopalan, K.V. (1996) Biosynthesis of the molybdenum cofactor. in *Escherichia coli and Salmonella typhimurium* (eds Neidhardt, F.C. et al) 2 nd ed. ASM Press, Washington, D.C. p.674-679.

Restrepo, M.A., Freed, D.D., and Carrington, J.C. (1990) Nuclear transport of plant potyviral proteins. *Plant Cell* 2: 987-998.

Reymond, P., and Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* 1: 404-411.

Riechmann, J.L., and Ratcliffe, O.J. (2000) A genomic perspective on plant transcription factors. *Curr. Opinion Plant Biol.* 3: 423-434.

Rossi, M., and Iusem, N.D. (1994) Tomato (*Lycopersicon esculentum*) genomic clone homologous to a gene encoding an abscisic acid-induced protein. Plant Physiol. 104: 1073-1074.

Sagi, M., and Lips, H.S. (1998) The levels of nitrate reductase and MoCo in annual ryegrass as affected by nitrate and ammonium nutrition. *Plant Sci.* 135: 17-24.

Sakurai, N., Hayakawa, T., Nakamura, T., and Yamaya, T. (1996) Changes in the cellular localization of cytosolic glutamine synthetase protein in vascular bundles of rice leaves at various stages of development. *Planta* 200: 306-311.

Samac, D.A., and Shah, D.M. (1991) Developmental and pathogen-induced activation of the Arabidopsis acidic chitinase promoter. *Plant Cell* 3: 1063-1072.

Sambroo,k J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.

Sarda, X., Tousch, D., Ferrare, K., Legrand, E., Dupuis, J.M., Casse-Delbart, F., and Lamaze, T. (1997) Two TIP-like genes encoding aquaporins are expressed in sunflower guard cells. *Plant J.* 12: 1103-1111.

Scheibel, T., Bell, S., and Walke, S. (1997) *S. cerevisiae* and sulfur: a unique way to deal with the environment. *FASEB J.* 11: 917-921.

Schindler, U., Beckmann, H., and Cashmore, A.R. (1992) TGA1 and G-box binding factors: two distinct classes of Arabidopsis leucine zipper proteins compete for the G-box-like element TGACGTGG. *Plant Cell* 4: 1309-1319.

Schneider, A., Salamini, F., and Gebhardt, C. (1997) Expression patterns and promoter activity of the cold-regulated gene ci21A of potato. *Plant Physiol.* 113: 335-345.

Schulze-Lefert, P., Becker-Andre, M., Schulz, W., Hahlbrock, K., and Dangl, J.L. (1989) Functional architecture of the light-responsive chalcone synthase promoter from parsley. *Plant Cell* 1: 707-714.

Schwarz, G., Schulze, J., Bittner, F., Eilers, T., Kuper, J., Bollmann, G., Nerlich, A., Brinkmann, H., and Mendel, R.R. (2000) The molybdenum cofactor biosynthetic protein Cnx1 complements molybdate-repairable mutants, transfers molybdenum to the metal binding pterin, and is associated with the cytoskeleton. *Plant Cell* 12: 2455-2472.

Senekoff, J.F., and Meagher, R.B. (1993) Isolating the *Arabidopsis thaliana* genes for *de novo* purine synthesis by suppression of *Escherichia coli* mutants. I. 5'-phosphoribosyl-5-aminoimidazole synthetase. *Plant Physiol.* 102: 387-399.

Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D.W. (1995) Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* 8: 777-784.

Shimada, Y., Wu, G-J., and Watanabe, A. (1998) A protein encoded by *din1*, a dark-inducible and senescence-associated gene of radish, can be imported by isolated chloroplasts and has sequence similarity to sulfide dehydrogenase and other small stress proteins. *Plant Cell Physiol.* 39: 139-143.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response *Plant Physiol.* 115: 327-334.

Silhavy, D., Hutvagner, G., Barta, E., and Banfalvi, Z. (1995) Isolation and characterization of a water-stress-inducible cDNA clone from Solanum chacoense. *Plant Mol. Biol.* 27: 587-595.

Singh, K., Dennis, E.S., Ellis, J.G., Llewellyn, D.J., Tokuhisa, J.G., Wahleithner, J.A., and

Peacock, W.J. (1990) OCSBF-1, a maize ocs enhancer binding factor: Isolation and expression during development. *Plant Cell* 2: 891-903.

Smart, C.M. (1994) Gene expression during leaf senescence. *New Physiol.* 126: 419-448.

Smart, C.M., Hosken, S.E., Thomas, H., Greaves, J.A., Blair, B.G., and Schuch, W. (1995) The timing of maize leaf senescence and characterisation of senescence-related cDNAs. *Physiol. Plant* 93: 673-682.

Smart, L.B., Cameron, K.D., and Bennett, A.B. (2000) Isolation of genes predominantly expressed in guard cells and epidermal cells of *Nicotiana glauca*. *Plant Mol. Biol.* 42: 857-869.

Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H., and Mendel, R.R. (1995) Molybdenum co-factor biosynthesis: the *Arabidopsis thaliana* cDNA *cnx1* encodes a multifunctional two-domain protein homologous to a mammalian neuroprotein, the insect protein Cinnamon and three *Escherichia coli* proteins. *Plant J.* 8: 751-762.

Stallmeyer, B., Schwarz, G., Schulze, J., Nerlich, A., Reiss, J., Kirsch, J., and Mendel, R.R. (1999) The neurotransmitter receptor-anchoring protein gephyrin reconstitutes molybdenum cofactor biosynthesis in bacteria, plants, and mammalian cells. *Proc. Natl. Acad. Sci. USA* 96: 1333-1338.

Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcription activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA* 94: 1035-1040.

Strathmann, A., Kuhlmann, M., Heinekamp, T., and Droge-Laser, W. (2001) BZI-1 specifically heterodimerises with the tobacco bZIP transcription factors BZI-2, BZI-3/TBZF and BZI-4, and is functionally involved in flower development. *Plant J.* 28: 397-408.

Tabata, T., Nakayama, T., Mikami, K., and Iwabuchi, M. (1991) HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat. *EMBO J.* 10: 1459-1467.

Taylor, C.B., Bariola, P.A., Del Cardayre, S.B., Raines, R.T., and Green, P.J. (1993) RNS2: a senescence-associated RNase of Arabidopsis that diverged from the S-RNases before speciation. *Proc. Natl. Acad. Sci. USA* 90: 5118-5122.

Taylor JE, Renwick KF, Webb AAR, McAinsh MR, Furini A, Bartels D, Quatrano RS, Marcotte Jr WR., and Hetherington AM (1995) ABA-regulated promoter activity in stomatal guard cells. *Plant J.* 7: 129-134.

Terryn, N., Arias, M.B., Engler, G., Tiré, C., Villarroel, Montagu, M.V., and Inzé, D. (1993) *rha1*, a gene encoding a small GTP binding protein from Arabidopsis, is expressed primarily in developing guard cells. *Plant Cell* 5: 1761-1769.

Thangavelu, M., Belostotsky, D., Bevan, M.W., Flavell, R.B., Rogers, H.J., and Lonsdale, D.M. (1993) Partial characterization of the *Nicotiana tabacum actin* gene family: evidence for pollen-specific expression of one of the gene family members. *Mol. Gen. Genet.* 240: 290-295.

Thomas, H., and Stoddart, J.L. (1980) Leaf senescence. *Annu. Rev. Plant Physiol.* 31: 83-111.

Thomas, H., and De Villiers, L. (1996) Gene expression in leaves of Arabidopsis thaliana induced to senesce by nutrient deprivation. *J. Exp. Bot.* 47: 1845-1852.

Thomas, T.L. (1993) Gene expression during plant embryogenesis and germination: an overview. *Plant Cell* 5:1401-1410.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876-4882.

Tournaire, C., Kushnir, S., Bauw, G., Inze, D., Teyssendier de la Serve, B., and Renaudin, J.P. (1996) A thiol protease and an anionic peroxidase are induced by lowering cytokinins during callus growth in Petunia. *Plant Physiol.*111: 159-68.

Twell, D., Yamaguchi, J., and McCormick, S. (1990) Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. *Development* 109: 705-713.

Twell, D., Yamaguchi, J., Wing ,R.A., Ushiba, J., and McCormick, S. (1991) Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev.* 5: 496-507.

Van Berkel, J., Salamini, F., and Gebhardt, C. (1994) Transcripts accumulating during cold storage of potato (*Solanum tuberosum* L.) tubers are sequence related to stress-responsive genes. *Plant Physiol.* 104: 445-452.

Van Gysel, A., Van Montagu, M., and Inze, D. (1993) A negatively light-regulated gene from Arabidopsis thaliana encodes a protein showing high similarity to blue copper-binding proteins. *Gene* 136: 79-85.

Vinson, C.R., Hai, T., and Boyd, S.M. (1993) Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev.* 7: 1047-1058.

Walker-Simmons, M., Kudrna, D.A., and Warner, R.L. (1989) Reduced accumulation of ABA during water stress in a molybdenum cofactor mutant of barley. *Plant Physiol.* 90: 728-733.

Walter, M.H., Liu, J.W., Wunn, J., and Hess, D. (1996) Bean ribonuclease-like pathogenesis-related protein genes (*Ypr10*) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. *Eur. J. Biochem.* 239: 281-293.

Wang, H., and Cutler, A.J. (1995) Promoters from *kin1* and *cor6.6*, two *Arabidopsis thaliana* low-temperature- and ABA-inducible genes: direct strong β-glucuronidase expression in guard cells, pollen and young developing seeds. *Plant Mol. Biol.* 28: 619-634.

Watanabe, A., Hamada, K., Yokoi, H., and Watanabe, A. (1994) Biphasic and differential expression of cytosolic glutamine synthetase genes of radish during seed germination and senescence of cotyledons. *Plant Mol. Biol.* 26: 1807-1817.

Weaver, L.M., Himelblau, E., and Amasino, R.M. (1997) Leaf senescence: gene expression and regulation. Genet. Eng. (NY) 19: 215-234.

Weaver, L.M., Gan, S., Quirino, B., and Amasino, R.M. (1998) A comparison of the expression patterns of several senescence associated genes in response to stress and hormone treatment. *Plant Mol. Biol.* 37: 455-469.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. *EMBO J.* 10: 1777-1786.

Weterings, K., Schrauwen, J., Wullems, G., and Twell, D. (1995) Functional dissection of the promoter of the pollen-specific gene *NTP303* reveals a novel pollen-specific, and conserved *cis*-regulatory element. *Plant J.* 8: 55-63.

Williams, M.E., Foster, R., and Chua, N.H. (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. *Plant Cell* 4: 485-496.

Willmer, C., and Fricker, M. (1996) Stomata (second edition), Topics in plant functional biology: 2, Chapman & Hall.

Xiang, C., Miao, Z., and Lam, E. (1997) DNA-binding properties, genomic organization and expression pattern of TGA6, a new member of the TGA family of bZIP transcription factors in *Arabidopsis thaliana*. *Plant Mol. Biol.* 34: 403-415.

Xiong, L., Ishitani, M., Lee, H., and Zhu, J-K. (2001) The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. *Plant Cell* 13: 2063-2083.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature or high-salt stress. *Plant Cell* 6: 251-264.

Yamamoto, Y.Y., and Deng, X.-W. (1998) A new vector set for GAL4-dependent

transactivation assay in plants. *Plant Biotech.* 15: 217-220.

Yanagisawa, S. (1998) Transcription factors in plants: physiological functions and regulation of expression. *J. Plant Res.* 111: 363-371.

Yanagisawa, S., and Schmidt, R.J. (1999) Diversity and similarity among recognition sequences of Dof transcription factors. *Plant J.* 17:209-214.

Yang, S.H., Berberich, T., Sano, H., and Kusano, T. (2001) Specific association of transcripts of *tbzF* and *tbz17*, tobacco genes encoding basic region leucine zipper-type transcriptional activators, with guard cells of senescing leaves and/or flowers. *Plant Physiol.* 127: 23-32.

Yoshida, T., and Minamikawa, T. (1996) Successive amino-terminal proteolysis of the large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase by vacuolar enzymes from French bean leaves. Eur. J. Biochem. 238: 317-324.

Zacarias, L., and Reid, M.S. (1990) Role of growth regulators in the senescence of Arabidopsis thaliana leaves. *Physiol. Plant.* 80: 549-554.

Zeevaart, J.A.D., and Creelman, R.A. (1988) Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* 39: 439-473.

Zhang, B., Foley, R.C., and Singh, K.B. (1993) Isolation and characterization of two related Arabidopsis *ocs*-element bZIP binding proteins. *Plant J.* 4: 711-716.

Zhang, B., Chen, W., Foley, R.C., Buttner, M., and Singh, K.B. (1995) Interactions between distinct types of DNA binding proteins enhance binding to *ocs*-element promoter sequences. *Plant Cell* 7: 2241-2252.

Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F. (2000) NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. Mol. Plant Microbe Interact. 13: 191-202.

Zimmer, W., and Mendel, R. (1999) Molybdenum metabolism in plants. *Plant Biol.* 1: 160-168.

List of publications

Seung Hwan Yang, Thomas Berberich, Hiroshi Sano and Tomonobu Kusano (2001) Specific association of transcripts of *tbzF* and *tbz17*, tobacco genes encoding bZIP-type transcriptional activators, with guard cells of senescing leaves and/or flowers. *Plant Physiol.*, 127: 23-32.

Seung Hwan Yang, Thomas Berberich, Hiroshi Sano and Tomonobu Kusano (2001) The darkness-induced and senescence-associated gene, *Ntdin*, encodes a protein functioning in molybdenum cofactor biosynthesis in tobacco plants. *Plant Science* (in press)

Seung Hwan Yang, Yube Yamaguchi, Nozomu Koizumi, Tomonobu Kusano and Hiroshi Sano (2001) Promoter analysis of *tbzF*, a gene encoding a bZIP-type transcription factor, reveals distinct variation in *cis*-regions responsible for transcriptional activation between senescing leaves and flower buds in tobacco plants. (submitted)

Acknowledgements

First of all, I would like to express my grateful appreciation to Professor Hiroshi Sano, Nara Institute of Science and Technology (NAIST), for giving me an opportunity to work in this research field and for his continuous guidance and encouragement throughout this study. I am also indebted to Professor Tomonobu Kusano, Tohoku university, and Dr. Thomas Berberich, J.W. Goethe-Universität, for their guidance and invaluable discussion throughout this research. I would like to thank Associate Professor Nozomu Koizumi and Dr. Yube Yamaguchi (NAIST). I would also like to express my thanks to Drs. Mikihiro Ogawa (RIKEN), Yoko Okushima (U. C. Berkeley), Tatsuo Nakamura (Yokohama National Univ.), Emiko Harada, Akira Nozawa, Rie Nishiyama and all other member of the Laboratory of Plant Molecular Breeding, Nara Institute of Science and Technology, for their useful discussion and an enjoyable laboratory life.

I express my sincere appreciation to Drs. Gyu-Hwa Chung, Sang-Yo Byun, Kong-Hwan Kim, Ill-Sup Nou, Kyun-Kyu Kang, Moon-Sik Hyun, Yong-Eui Choi and Dae-Jin Yun for valuable suggestions and encouragement.

I appreciate very much for the Japanese Government (Monbusho) to provide me a fellowship giving the opportunity to accomplish Ph.D thesis in Japan.

Finally, my deepest thanks are given to my wife, Guenjung, for her encouragement and support. Special thanks are due to my parents, parents in law, my brother, my sister, my relatives and my friends for their support in many ways. A new born my daughter, Suhyun, has been an inspiration to me throughout my studies. Thank God for his love.