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Title: Transformation of Tobacco with the Yeast *FRE1* and *FRE2* genes: Characterization of Transformants and Discovery of a Temperature-Dependent Morphological Mutant.

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A key mechanism utilized by plants to make iron (Fe) available for uptake is the reduction of Fe(III) to Fe(II) via an inducible, plasma membrane-bound Fe(III) reductase. Genes encoding such enzymes have not yet been isolated from plants; however, two Fe(III) reductases have been cloned from yeast. *FRE1* and *FRE2* account for the total membrane-associated Fe(III) reductase activity in *Saccharomyces cerevisiae*. If yeast reductase genes could be expressed in a plant system, root Fe(III) reduction may be enhanced, leading to a decrease in Fe chlorosis in transgenic plants.

*FRE1* and *FRE2* were introduced into tobacco via *Agrobacterium*-mediated transformation. Fe(III) reductase activity was measured in homozygous transformants containing *FRE1*, *FRE2*, or both. The highest Fe(III) reduction levels were found in lines containing both *FRE1* and *FRE2*. Liquid reductase assays showed three to four times more Fe(III) reduction in these transformants as compared to controls, and visual plate assays

showed reduction along the entire length of the roots. One *FRE1*-containing line initially exhibited chlorosis on medium with low Fe at pH 7.5, but later recovered. Other transformants and the control remained chlorotic.

*Agrobacterium*-mediated transformation often produces mutant phenotypes. A temperature-dependent morphological mutant was found among the progeny of tobacco transformed by *Agrobacterium*. The mutation is recessive and is expressed at low temperature (21°C). Mutant characteristics include formation of thick, narrow leaves with abnormal mesophyll cells and near absence of apical dominance. Also in the greenhouse (21-23°C), most plants remain vegetative, and the few flowers that are formed have petaloid stamens. High temperature (30°C) reverses the mutant phenotype, with formation of normal leaves and restoration of apical dominance. However, many flowers still have petaloid stamens. This mutant shares several phenotypic characteristics with transgenic tobacco plants overexpressing maize and *Arabidopsis* homeodomain proteins.

# Transformation of Tobacco with the Yeast *FRE1* and *FRE2* genes: Characterization of Transformants and Discovery of a Temperature-Dependent Morphological Mutant

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TABLE OF CONTENTS

Page

INTRODUCTION AND LITERATURE REVIEW . . . . . 1

Introduction . . . . . 1

Literature Review . . . . . 3

Fe nutrition in plants . . . . . 3

Importance of Fe . . . . . 3
Morphological responses to low Fe . . . . . 4
Physiological responses to low Fe . . . . . 5
Plant Fe(III) reductases . . . . . 6

Fe nutrition in yeast . . . . . 11

Yeast Fe(III) reductases . . . . . 11
The Copper-Iron connection . . . . . 17

Complementation with heterologous genes . . . . . 18

Morphological mutants . . . . . 21

Altered stearate levels . . . . . 21
Overexpression of cytokinins . . . . . 22
The Schizoid mutant . . . . . 23
MADS box proteins . . . . . 24
Homeodomain proteins . . . . . 24
MADS/homeodomain interactions . . . . . 26

EXPRESSION OF YEAST *FRE1* AND *FRE2* GENES IN TOBACCO . . . 28

Abstract . . . . . 28

Introduction . . . . . 28

Materials and Methods . . . . . 31

PCR of *FRE1* . . . . . 31
Cloning of *FRE1* into Plasmids . . . . . 31
PCR of *FRE2* . . . . . 33
Cloning of *FRE2* into Plasmids . . . . . 33
Sequencing of *FRE1* . . . . . 34

TABLE OF CONTENTS (continued)

Page

*In Vitro* Transcription/Translation of *FRE1* . . . . . 34
Transformation of *Agrobacterium* . . . . . 34
Transformation of Tobacco . . . . . 35
Scoring of Transgenic Progeny . . . . . 36
Southern and Northern Blotting . . . . . 36
Fe Reductase Assay . . . . . 38
Response to Fe-Deficient Medium . . . . . 39

Results . . . . . 40

In Vitro Transcription/Translation . . . . . 40
Tobacco Transformants . . . . . 41
Southern and Northern Analyses . . . . . 44
Fe(III) Reduction . . . . . 47
Growth Responses to Fe-Deficient Medium . . . . . 54

Discussion . . . . . 61

A TEMPERATURE-DEPENDENT MORPHOLOGICAL MUTANT OF TOBACCO . . . . . 64

Abstract . . . . . 64

Introduction . . . . . 65

Materials and Methods . . . . . 66

Origin of the Mutant . . . . . 66
Growth Analyses Under Controlled Environment . . . . . 67
Anatomy . . . . . 68
Fatty Acid Analysis . . . . . 68
Grafting . . . . . 69
Effects of Cytokinin and Auxin . . . . . 69
T-DNA Analyses . . . . . 70

Results . . . . . 70

Origin and inheritance . . . . . 70
Morphology . . . . . 73
Effects of temperature . . . . . 73
Leaf anatomy . . . . . 81
Grafting, growth regulators, and fatty acids . . . . . 81

TABLE OF CONTENTS (continued)

Page

Search for T-DNA . . . . . 86

Discussion . . . . . 88

SUMMARY AND CONCLUSIONS . . . . . 92

BIBLIOGRAPHY . . . . . 95

LIST OF FIGURES

Figure — Page

2.1. *In vitro* transcription/translation of *FRE1* without canine microsomes (lane 1) and with microsomes (lane 2) . . . . . 42

2.2. Southern blots of *Eco*RV-digested tobacco genomic DNA . . . . 45

2.3. PCR using *FRE2* border primers . . . . . 48

2.4. Northern blots . . . . . 50

2.5. Root Fe(III) reduction in lines with *FRE1*, *FRE2* and *FRE1+2* . . 52

2.6. Root Fe(III) reduction in lines containing *FRE1+2* . . . . . 55

2.7. Fe(III) reduction visualized by embedding seedlings in medium containing Fe(III) and BPDS for 6 h . . . . . 57

2.8. Tobacco seedlings grown on high pH, low Fe medium for 8 weeks . . . . . 59

3.1 A-F. Greenhouse-grown *tds* mutant (**A**), multiple meristems on *tds* shoot apex (**B**), flowers of *tds* (left) and WT (right) with the corolla removed (**C**), *tds* flower showing corolla and petaloid stamens (**D**), plants grown in growth chambers for 49 d, from left to right: WT at 30°C, *tds* at 30°C, WT at 21°C, and *tds* at 21°C (**E**), and *tds* plant grown at 30°C and then 21°C (**F**) . . . . . 71

3.2. Longitudinal sections of *tds* shoot apex showing multiple meristems; x 24 (**A**), WT shoot apex; x 24 (**B**), and various types of *tds* shoot apices; x 76 (**C-E**). Bar = 100 $\mu$m. . . . . 74

3.3 A, B. *tds* seedlings in Magenta boxes after 27 d at 28°C (**A**), and 21°C (**B**) . . . . . 77

3.4. Plant height of WT and *tds* grown at 21°C and 30°C . . . . . 79

3.5. WT (left) and *tds* (right) leaves photographed on a light box . . 82

3.6 A-F. Cross sections of leaves from *tds* (**A,C,E**) and WT (**B,D,F**) 84

# Transformation of Tobacco with the Yeast *FRE1* and *FRE2* genes: Characterization of Transformants and Discovery of a Temperature-Dependent Morphological Mutant

## CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

### Introduction

Many plants are sensitive to low iron (Fe) conditions, especially fruit trees such as pear and peach (Faust, 1989). A solution to alleviate Fe-deficiency chlorosis is the application of Fe-chelates. This approach is expensive and contributes to groundwater contamination, while providing only temporary relief (Wallace, 1983). Genetic improvement offers a more permanent solution, but breeding of fruit trees is complicated by high heterozygosity and a long juvenile phase. Therefore, biotechnology may play an important role in enhancing Fe efficiency by employing heterologous genes which aid in Fe utilization.

Fe in the soil often exists as insoluble ferric (Fe(III)) oxides and hydroxides which can not be utilized by plants. A key mechanism for increasing the Fe availability is reduction of Fe(III) to Fe(II), which can then be taken up by the roots (Chaney et al., 1972). This reaction is mediated by Fe(III) reductases. Thus, transforming plants with genes encoding Fe(III) reductases may be an effective strategy to enhance Fe availability and utilization.

Although plant Fe(III) reductases have been partially purified from plasma membranes, the corresponding genes have yet to be isolated. However, two genes involved in similar processes in the yeast *Saccharomyces cerevisiae*, *FRE1* and *FRE2*, have been cloned (Dancis et al., 1992; Georgatsou and Alexandraki, 1994). As in plants, the yeast Fe uptake system involves reductase activity acting on Fe(III) chelates external to the cell. *FRE1* was first isolated by mutant complementation (Dancis et al., 1992). Transformation of yeast with a plasmid containing *FRE1* resulted in a three-fold increase in reductase activity and restored normal growth (Dancis et al., 1992). The second gene, *FRE2*, was discovered during the course of the European Community project on yeast chromosome XI sequencing. Georgatsou and Alexandraki (1994) showed that the combination of *FRE1* and *FRE2* accounted for the total membrane-associated Fe(III) reductase activity in yeast. The utility of these yeast genes in enhancing Fe efficiency in plants is tested in this study. Tobacco is utilized as a model system for expression of the yeast Fe(III) reductases, with the ultimate goal of introducing useful clones into other plants, such as fruit trees. Construction and analyses of transgenic tobacco plants containing *FRE1*, *FRE2*, and *FRE1* + *FRE2* are described in Chapter II.

A temperature-dependent morphological mutant was identified among the selfed progeny of a primary transformant involving *FRE1*. Segregation patterns indicated that the mutant is recessive and not related

to the presence of *FRE1*. The mutant phenotype, characterized by multiple shoots with thick, narrow leaves, and abnormal mesophyll cells, is expressed only at low temperature (18-21°C). At high temperature (30°C), development is normal with the exception of petaloid stamens. The mutant is described in Chapter III.

## Literature Review

### Fe nutrition in plants

#### Importance of Fe

Fe is an essential micronutrient required for normal functioning of plant cells. Fe-containing enzymes participate in nitrogen reduction, respiration, photosynthesis, and chlorophyll biosynthesis. In the absence of adequate Fe supplies, interveinal chlorosis occurs. Excess Fe accumulation can be toxic due to stimulation of free radical reactions (Halliwell and Gurreridge, 1988). Consequently, Fe uptake and storage are highly regulated processes.

Even though Fe is the fourth most abundant mineral in the earth's crust, it is often unavailable to plants growing in aerated and alkaline soils. These soils contain predominantly insoluble Fe(III) oxides and hydroxides. Suboptimal Fe concentrations can result in loss of crop yield (Lindsay and Schwab, 1982). Free Fe(II) ions are readily taken up by roots (Chaney et al., 1972). Organisms typically utilize three mechanisms to dissolve Fe(III)

oxides: chelation, protonation, and reduction (Romheld, 1987; Guerinot and Yi, 1994).

Dicots and nongraminaceous monocots (Strategy I plants) share Fe uptake mechanisms with yeast. Fe(III) is first solubilized by reduction, and then the resulting Fe(II) is carried across the plasma membrane (PM) via a specific Fe(II) transporter (Guerinot and Yi, 1994). Graminaceous monocots (Strategy II plants), bacteria, and certain fungi utilize different mechanisms. Fe(III) is first solubilized by binding secreted high-affinity chelators called siderophores (phytosiderophores in plants), which are then recognized by specific membrane receptors. The Fe(III) complexes are internalized and Fe is separated from the siderophore. Strategy I plants and yeast will be emphasized in the sections that follow. Strategy I plants demonstrate both morphological and physiological modifications when the Fe supply is limited (Romheld, 1987).

Morphological responses to low Fe

Changes in morphology include increased lateral root formation and development of specialized rhizodermal transfer cells in the apical zones of the roots (Romheld, 1987). Transfer cells have labyrinth-like invaginations which serve to increase the surface area of the PM. Most dicot roots have a short region, a few mm behind the tip and directly behind the elongating zone, where Fe(III) chelates are reduced. Upon Fe deficiency, this zone can be extended to be several cm long. Fe(III) reduction is restricted to the

outer layer of cells (epidermis) of the roots. Additionally, under Fe deficiency, root apical zones are often swollen and have an enhanced number and increased length of root hairs (Landsberg, 1982). These changes serve to increase the surface area for reduction and transport of Fe. Moog et al. (1995) examined Fe deficiency responses in an *Arabidopsis* root hair-less mutant RM57 (Schiefelbein and Somerville, 1990). This mutant did not develop transfer cells upon exposure to an Fe-deficient medium and, with respect to root morphology, showed identical responses to an Fe-free medium as the wild type, except for lack of root hair formation. Moog et al. (1995) concluded that there are at least two different detection systems in the plant for the Fe status of the root: one for the morphological, and one for the physiological response.

Physiological responses to low Fe

One important physiological response to low Fe availability is the increased acidification of the apoplast and rhizosphere through enhanced proton extrusion by roots (Romheld and Marschner, 1984; Romheld et al., 1984). This decrease in pH, brought about by $H^+$-ATPases, can greatly enhance the solubility of Fe(III), resulting in enhanced Fe uptake. $H^+$-ATPases are involved in many physiological processes (reviewed in Serrano, 1988 & 1989; Michelet and Boutry, 1995) and are encoded by several genes (Sussman, 1994). Serrano calls them "master enzymes" since they control a number of very important functions at the cellular level, such as

cell division and elongation as well as ion transport. The existence of $H^+$-ATPase gene families permits fine regulation of $H^+$-ATPase activity in different cells and tissues (Michelet and Boutry, 1995).

Another physiological response is the secretion of reducing compounds by root systems. Chaney et al. (1972) discovered that compounds capable of reducing Fe(III) chelates were excreted from roots. High levels of phenolics and organic acids (citrate and malate) were found in roots of Fe-deficient plants (Brown and Ambler, 1973; Landsberg, 1981; Olsen and Brown, 1980; Olsen et al., 1981; Romheld and Marschner, 1981 & 1983). These substances can help mobilize inorganic Fe(III) through acidification, chelation and reduction.

Reduction of Fe(III) to Fe(II) via an inducible, PM-bound Fe(III) reductase is a key factor in making Fe available for absorption. All three adaptation mechanisms can lead to increased Fe acquisition and often they are expressed together under Fe-limiting conditions (Romheld, 1987). However, Fe reductases may play a particularly important role and are discussed in more detail.

Plant Fe(III) reductases

Based on experiments using intact roots, Bienfait (1985) proposed a model in which two types of PM bound redox systems are discerned: the so-called "standard" (constitutive) and "turbo" (inducible) systems (reviewed in Bienfait, 1985 & 1988 & 1989; Bottger et al., 1991; Chaney

and Bell, 1987). According to this model, the standard reductase, found in the PM of all growing cells, reduces Fe(III) complexed in high potential electron acceptors such as ferricyanide. This enzyme does not depend on the Fe status of the plant. Ferricyanide is an artificial electron acceptor which, when reduced, produces ferrocyanide (neither form is accessible to transmembrane uptake). This compound is utilized as a convenient way to demonstrate differences between reductases in a laboratory setting. The standard reductase may not be directly involved in Fe uptake, but may serve a more general regulatory function in ion-transport processes (i.e. for the integrity of PM redox systems) (Bienfait and Luttge, 1988). The turbo reductase, induced in root cells by Fe deficiency, has low specificity with regard to substrate and is capable of reducing both ferricyanide and various Fe(III) chelates. This reductase is thought to be responsible for generating Fe(II) for subsequent transport across the PM of root cells.

Advanced membrane purification techniques have allowed researchers to isolate purified PM without contaminants (Larsson and Moller, 1990). Challenges to Bienfait's model have arisen from work with Fe reductases isolated from tomato root PM (Bruggemann et al., 1990; Holden et al., 1991). Research findings could not support the existence of two types of Fe reductases, in particular the turbo reductase. The increase in reductase activity appeared to reflect enhanced expression of the constitutive reductase, since Fe stress resulted in higher intensities of isozyme bands already present in PM extracts of Fe-sufficient roots. This

led to revised definitions and a new working model was proposed (Moog and Bruggemann, 1994).

The model of Moog and Bruggemann (1994) states that the standard system of the root PM involves at least two constitutive transmembrane Fe(III) reductase activities, which can reduce external electron acceptors such as ferricyanide and Fe(III) chelates. These reductases utilize either NADH or NADPH as electron donors and oxygen and Fe(III) chelates as the natural electron acceptors. Biologically, the standard reductase is involved in Fe uptake under Fe-sufficient conditions and possibly other functions such as membrane polarization and control of cell elongation or proliferation (Moog and Bruggemann, 1994). The activity of the NADH-dependent constitutive Fe reductase increases upon Fe deficiency, either by enzyme activation or through induced protein synthesis. Inducible redox activity is located on the PM in distinct root zones of Strategy I plants. This enzyme reduces apoplastic ferricyanide and Fe(III) chelates using electrons delivered by cytoplasmic NADH and the physiological function is to mobilize Fe for uptake by the roots.

Holden et al. (1994) have partially purified the Fe(III) chelate reductase from the PM of Fe-deficient tomato roots. Using a Procion Red Sepharose affinity column, a single peak of Fe(III) chelate reductase activity was eluted near the end of a simultaneous gradient of NADH and KCl. Also, two peaks of NADH-dependent ferricyanide reductase activity were found; one coinciding with the Fe(III) chelate reductase activity, the other

eluting during column washing prior to gradient elution. This provided further evidence for more than one electron transport activity in the root PM: one using Fe(III) chelates and ferricyanide as electron acceptors, the other reducing only ferricyanide. Previous work utilizing preparative isoelectric focusing demonstrated ferricyanide reductase activity with two different isoelectric points, only one of which was contiguous with the Fe(III) reductase isoforms (Holden et al., 1991). Using a chromatofocusing column as another purification step for Fe(III) chelate reductase, a polypeptide of 34 to 36 kDa correlated well with the activity. Since other polypeptides were also associated with the Fe(III) chelate reductase (but were not active) and many electron transport systems consist of multiple components, the tomato root Fe(III) chelate reductase enzyme may be comprised of more than one polypeptide. Holden et al. (1994) postulate that the 35 kDa polypeptide is a catalytic component of the Fe(III) chelate reductase. Further analyses revealed that the Fe(III) chelate reductase does not have glycosylation sites, nor is it a heme-containing cytochrome.

Recently, Susin et al. (1996) examined Fe reduction in beets, which are extremely efficient Strategy I plants. They proposed that mechanisms resulting from Fe-stress conditions under low pH may differ from those resulting from high pH, and lend support to Bienfait's turbo model involving induction of a new Fe reductase. In contrast to the small increase in Fe(III) chelate reductase activity at high pH values, Fe-deficient intact plants assayed at pH 6.0 or below exhibited 19-fold (turbo) increases in reductase

activity. It was suggested that the mechanism responsible for the turbo Fe(III) chelate reductase activity in Fe-deficient, intact plants at pH 6.0 or below was different from that present at higher pH values. The Fe(III) chelate reductase activity in Fe-starved, intact plants at pH 6.5 or above was biochemically similar to the constitutive Fe(III) chelate reductase present in the control plants at any pH.

A possible alternative to biosynthesis of a distinct enzyme is that under Fe-deficient conditions the standard Fe(III) chelate reductase may undergo a modification that increases its capacity to reduce Fe (Susin et al., 1996). A key question yet to be resolved is why a turbo-type Fe(III) reductase activity is detected in intact plants, but cannot be observed in PM preparations. Future experiments will test the hypothesis that an as-yet-unidentified cofactor, necessary for the functioning of the turbo Fe(III) chelate reductase, may be lost during the preparation of PM fractions (Susin et al., 1996).

The ability of PM to reduce ferricyanide appears to be an ubiquitous feature of higher plant cells, since such activities have been reported for stem, leaf and flower tissues (Moog and Bruggemann, 1994). Since the transported form of Fe in the xylem is ferric citrate (Brown and Jolley, 1986), the question was raised whether Fe uptake by leaf cells may be mediated by a PM redox system akin to that in the roots (Bienfait, 1989; Bottger et al., 1991). Bruggemann et al. (1993) gave the first evidence of such a redox system in the PM of *Vigna unguiculata* mesophyll cells. Using

uptake of $^{59}Fe$ from various Fe(III) sources (including $^{59}Fe$(III)[$^{14}C$]citrate), they demonstrated that uptake depended on an obligatory reduction step of Fe(III) to Fe(II). The physiological role of Fe(III) chelate reductase and ferricyanide reductase for Fe uptake and acquisition in non-root tissue is still an open question.

Welch et al. (1993) suggested that the Fe(III) reductase may play a more general role in regulating cation uptake. Stimulation of reductase activity via Fe-deficiency also significantly increased accumulation of a number of mineral cations (i.e. Cu, Mn, Fe, Mg and K). Roots from both Fe-deficient and Cu-deficient plants reduced exogenous Cu(II) chelate as well as Fe(III) chelate. Root PM reductases may additionally control the reduction of critical sulfhydryl groups in proteins involved in divalent cation transport across the root PM.

**Fe nutrition in yeast**

Yeast Fe(III) reductases

*Saccharomyces cerevisiae* does not secrete siderophores (Neilands et al., 1987), but it can reduce extracellular Fe(III) chelates, including Fe bound to siderophores produced by other organisms, through a PM redox system that is induced in Fe-deficient conditions. Fe(II) ions are then taken up by the cell (Lesuisse et al., 1987). This yeast is also able to assimilate Fe from the siderophore ferrioxamine B through a non-reductive process involving internalization of the siderophore (Lesuisse and Labbe, 1989).

Afterwards, Fe is released inside the cell, probably via a reduction step. Lesuisse et al.(1990) examined subcellular fractions of *S. cerevisiae* and identified and characterized the Fe(III) reductases involved in Fe assimilation. When Fe(III)EDTA was used as an electron acceptor, both NADH- and NADPH-dependent Fe(III) reductase activities were detectable. The highest NADPH-dependent activity was found in the PM fraction, while NADH-dependent activity was identified in the crude membrane fraction (consisting mostly of mitochondrial membranes). When ferrioxamine B was used as the Fe(III) substrate, NADH-dependent Fe(III) reductase activity was low in all fractions and NADPH was the ideal electron donor. Correlation of activities with different subcellular fractions indicated that several Fe-releasing enzymes were required for Fe assimilation *in vivo*. Attempts to purify the PM Fe(III) reductase to homogeneity resulted in complete loss of activity. It was suggested that several components, rather than a single protein, could be involved and therefore, activity dropped with loss of subunits upon purification. This enzyme is a flavoprotein with a weakly bound FMN prosthetic group; it has a high affinity for NADPH and a pH optimum at 7.5. The enzyme has low substrate specificity, matching previous reports for *in vivo* studies in which whole cells reduced Fe(III) citrate, Fe(III) EDTA, and several Fe(III) siderophores (Lesuisse et al., 1987; Lesuisse and Labbe, 1989). Heme-deficient yeast mutants lacked inducible Fe(III) reductase activity and were unable to take up Fe from different Fe(III) chelators such as Fe(III) citrate and rhodotorulic acid (Lesuisse and Labbe,

1989). However, ferrioxamine B was actively taken up by both mutant and wild type strains. This led to conclusions that complex mechanisms are involved in yeast Fe uptake and heme is required for development of Fe(III) reductase activity.

Dancis et al. (1990) mutagenized *S. cerevisiae* with ethylmethanesulfonate (EMS) and isolated a mutant lacking externally directed PM reductase activity. This mutation could be complemented with wild type genomic DNA fragments in a YCp50 shuttle vector. The *FRE1* gene was subsequently cloned (Dancis et al., 1992). Genetic analysis of this mutant yeast (*fre1-1*) revealed that both the reductase and Fe uptake deficiencies were due to a single mutation. The *FRE1* gene is a single open reading frame (ORF) of 2061 base pairs and the predicted protein of 686 amino acids has a calculated molecular mass of 78.8 kDa (Dancis et al., 1992). There are six potential sites for the addition of N-linked sugars and the first 22 amino acids match the von Heijne consensus for the leader peptide of a membrane or secreted protein (von Heijne, 1983). The protein has two amino-terminal hydrophobic regions that are strong candidates for transmembrane domains and five other hydrophobic regions which may also span the membrane. FRE1 has limited homology (17.9% identity and 62.2% similarity over the carboxy-terminal 402 amino acids) to the large subunit of human cytochrome $b_{558}$ (gp91-*phox* protein), the protein affected in the X-linked form of chronic granulomatous disease (CGD) (Orkin, 1989). FRE1 and the human granulocyte reductase are most similar in regions of

the protein thought to be involved in the binding of flavin adenine dinucleotide and NADPH (Roman et al., 1993).

It was suggested that the presence of a functional Fe(II) uptake system in *fre1-1* might explain viability of these cells in Fe-rich media. Reduction of some Fe(III) either by chemical means, or by residual reductase activity may have supplied enough Fe(II) to allow for survival. It was concluded that Fe uptake in *S. cerevisiae* is mediated by two PM components: a reductase and a Fe(II) transport system. Eide et al. (1992) further demonstrated that the Fe(III) reductase and Fe(II) transporter are separately regulated and that Fe accumulation may be limited by changes in either activity.

Dancis et al. (1992) fused 977 base pairs of the 5′ noncoding region and the first three codons of *FRE1* with the *E. coli* lacZ gene on a high-copy-number plasmid. This gave ß-galactosidase activity in wild-type cells that was regulated by the Fe content of the growth medium, showing 55-fold higher expression in Fe-depleted medium. Since the range of regulation of ß-galactosidase activity from this construct was similar to that seen for Fe(III) reductase, it was concluded that regulation of *FRE1* expression by Fe is at the level of transcription. It was suggested that *FRE1* may not encode the only reductase utilized by *S. cerevisiae* in Fe reduction and uptake. This assessment was based on the observation that there was residual Fe(III) reductase and Fe uptake in their *FRE1* deletion/disruption mutant strains. The ability of high concentrations of Fe(III) in the medium

to completely correct the growth deficiency of the mutants suggested the existence of an alternative uptake system, perhaps one of lower affinity than that involving FRE1.

A similar study was carried out in the fission yeast *Schizosaccharomyces pombe* (Roman et al., 1993). This resulted in cloning of the *frp1*+ gene, which shows similarity to *FRE1* and the human gp91-*phox* gene. Promoter-reporter gene fusions demonstrated mRNA repression in the presence of Fe and activation upon Fe starvation. Frp1 showed 27% amino acid identity and 49% similarity to FRE1 and 20% amino acid identity and 48% similarity to gp91-phox. Perhaps the most useful comparison was that of the hydropathy profiles of the three proteins (Roman et al., 1993) in which five conserved motifs are located in similar positions in the carboxy-terminal region. It was concluded that in comparing the Fe uptake systems of *S. cerevisiae* and *S. pombe*, there are basic similarities of structure and regulation, but limited sequence conservation.

Georgatsou and Alexandraki (1994) cloned *FRE2*, a gene which was shown to account, together with *FRE1*, for the total membrane-associated Fe(III) reductase activity in *S. cerevisiae*. This gene was identified during the sequencing of yeast chromosome XI, revealing an ORF of 711 codons whose putative protein product showed similarity (24.5% identity in 693 overlapping amino acids) to FRE1. The amino terminus of FRE2 has the von Heijne consensus for a leader peptide of membrane or secreted proteins

(von Heijne, 1983). Both its hydrophobicity profile and potential sites for addition of N-linked sugars correlate well with those of FRE1.

Yeast deletion mutants were created for *FRE1*, *FRE2* and *FRE1* + *FRE2* (Georgatsou and Alexandraki, 1994). Deletion of both *FRE1* and *FRE2* genes completely abolished the membrane-associated Fe(III) reductase activity, rendering the cell incapable of growing for an extended period in Fe-deficient media. Separate deletions of *FRE1* or *FRE2* did not drastically alter the levels of reductase activity, indicating that each gene product could qualitatively substitute for the other. Analysis of growing yeast cultures demonstrated that FRE2 accounted for at least 80% of the reductase activity after 12h of growth in Fe-deficient media, whereas FRE1 is responsible for more than 65% of the wild type activity after 3 to 4h of growth. Thus, these proteins have different roles at different phases of cell growth. Perhaps unique cis-acting elements contribute to the transcription of the two genes, accounting for their differential regulation. This is supported by the fact that none of the elements identified by Dancis et al. (1992) in the *FRE1* promoter exist in the 156 bp promoter region of *FRE2* (or within 1 kb upstream of the initiator AUG). Even though *FRE1* and *FRE2* have the same enzymatic function, they do not show significant similarity at the nucleotide level, and their products have borderline similarity in amino acid sequence. This suggests that the two genes have probably derived from a common ancestral gene following extensive

nucleotide divergence; presumably the only preserved sequences are those that ensure their enzymatic function and membrane localization.

The Copper-Iron connection

Two different genes were identified which linked Cu and Fe uptake (Askwith et al., 1994; Dancis et al., 1994). FET3 encodes a Cu-dependent ferro-oxidase (Askwith et al., 1994) and CTR1 encodes a Cu transporter (Dancis et al., 1994). It was soon discovered that transport of Fe(II) in *S. cerevisiae* not only depends on reduction of Fe(III) in the extracellular milieu, but is also coupled to re-oxidation of Fe(II) (Chang and Fink, 1994). Recently Stearman et al. (1996) cloned *FTR1*, the yeast Fe transporter, and provided evidence that a complex forms between FTR1 and FET3. According to the current model, the PM-bound CTR1 protein transports Cu into the cell. Among other things, this Cu becomes a key component of a Fe(II) oxidase (encoded by the *FET3* gene). FET3 has an external multicopper oxidase domain tethered to the PM by a single transmembrane unit (Kaplan & O'Halloran, 1996). FRE1 and FRE2 reduce extracellular Fe(III) to Fe(II), and then FET3 catalyzes conversion of Fe(II) to Fe(III). Fe(III) is immediately passed to the associated FTR1 transmembrane transporter, which allows Fe to enter the cell. A key question remains as to why catalytic re-oxidation of Fe(II) to Fe(III) is necessary for transport. FRE1 is linked to both Cu and Fe metabolism, since its transcription is repressed not only by Fe, but also by Cu (Jungmann et al., 1993). The Cu

and Fe effects on *FRE1* expression are controlled by a novel transcription factor, MAC1 (Jungmann et al., 1993). FRE1 can also serve as a Cu reductase. However, it still is uncertain whether oxidation or reduction is required for Cu transport.

**Complementation with heterologous genes**

Frommer and Ninnemann (1995) have reviewed the heterologous expression of genes in bacterial, fungal, animal and plant cells, with emphasis placed on membrane proteins. They point out that the sequences of most eukaryotic proteins are well conserved. Eukaryotes have several processes in common, including principles of cell compartmentation, intracellular transport and regulation, such as vesicular trafficking through the secretory pathway, cell-cycle control, signal transduction, and chromatin structure. Important differences also exist between fungal, plant, and animal cells regarding the presence and composition of cell walls and use of specialized organelles such as plastids and vacuoles. In terms of energization of secondary active transport processes at the PM, plants are more similar to yeast than animal cells: plants and yeast utilize proton gradients, whereas animals use mainly sodium gradients. Many properties are unique to multicellular organisms and no equivalent exists in unicellular organisms. These include intercellular communication across cell walls and through signals carried in the vascular system of plants, or electrical and hormonal long-distance communication in animals.

Yeast has become the preferred expression system for plant membrane proteins and many genes have been identified via complementation of yeast mutants (Frommer and Ninnemann, 1995). Proteins are processed and often targeted correctly to the PM of yeast. The *Arabidopsis thaliana* $H^+$-ATPase AHA2 was able to partially complement the *S. cerevisiae* ATPase mutant *pma1* (Villalba et al., 1992). The protein was functional but most of the plant ATPase was not expressed in the yeast PM. Instead, the enzyme remained trapped at a very early stage of the secretory pathway: insertion into the endoplasmic reticulum. Later on, removal of the C-terminal domain of the plant protein led to increased targeting to the PM and fully complemented *pma1*. Addition of yeast targeting sequences can improve heterologous expression. Complementation involves a single introduced cDNA; therefore, one cannot identify genes encoding polypeptides that are subunits of multimeric proteins or that are dependent on specific cofactors.

Recently, Eide et al. (1996) provided the first molecular insight into Fe transport in plants by complementing a yeast strain defective in Fe uptake. A *fet3 fet4* yeast double mutant was transformed with an *Arabidopsis* cDNA library, and mutants surviving on Fe-limited media were identified. This led to cloning of the *IRT1* (iron-regulated transporter) gene, which is predicted to encode an integral membrane protein with a metal-binding domain. In *Arabidopsis*, *IRT1* is expressed in roots, and is induced

by Fe deficiency. Data base comparisons showed related sequences in the genomes of rice, yeast, nematodes, and humans.

Although a large number of plant genes have been expressed in yeast, only a couple of yeast genes have been expressed in plants. The *S. cerevisiae ILV1* gene, encoding threonine dehydratase (localized in the mitochondrion) was able to complement an isoleucine-requiring *Nicotiana plumbaginifolia* auxotroph deficient in threonine dehydratase (Colau et al., 1987). The yeast gene was driven by the nopaline synthase promoter and the plant-produced protein was similar to yeast ILV1. This led to the conclusion that plant cells can recognize and cleave the amino-terminal leader sequence of the ILV1 primary translation product involved in the transport into yeast mitochondria. Von Schaewen and co-workers (1990) modified the yeast invertase gene (*suc2*), which hydrolyzes sucrose to glucose and fructose, by adding N-terminal signal peptides from the potato-derived vacuolar protein proteinase inhibitor II. With a 35S CaMV promoter driving this construct, the yeast invertase was secreted into the cell wall and was functional. This apoplastic protein served to interrupt sucrose export and led to an accumulation of carbohydrates, plus inhibition of photosynthesis, in transgenic plants. Ow cloned a yeast gene which produces a peptide that carries a cadmium-binding phytochelatin into the vacuoles (Moffat, 1995). He has successfully transformed both tobacco and *Arabidopsis* with this gene. Unfortunately, plant cells were unable to express the yeast gene and no cadmium tolerance was observed. Ow

suggests there may be a problem with certain DNA sequences within the yeast gene and hopes to solve it by modifying the gene and its regulatory sequences.

**Morphological mutants**

Plant morphological mutants result from a wide variety of genetic lesions and can arise naturally, through chemical mutagens (McHale, 1992), through insertional mutagenesis via transposons (Hake et al., 1989) or T-DNA (Feldmann, 1991), and through ectopic expression of transgenes (Sinha et al., 1993; Aoyama et al., 1995; Uberlacker et al., 1996). Aberrations might be a consequence of a number of disruptions. The large number of morphological mutants isolated over the years make it impossible to describe each in detail. Due to the similarities between the *tds* mutant described in chapter III, the following review will focus on mutants or transformants resulting in overproduction of fatty acids (Lightner et al., 1994), hormones (Klee et al., 1987; Medford et al., 1989; Estruch et al., 1991), or putative transcription factors (Kempin et al., 1993; Sinha et al., 1993; Aoyama et al., 1995; Uberlacker et al., 1996).

Altered stearate levels

Lightner et al. (1994) identified the *fab2* mutant of *Arabidopsis thaliana* by screening an EMS mutagenized population. The *fab2* mutant is characterized by its miniature form, correlated with an increased level of the fatty acid stearate (18:0), a constituent of membrane lipids. Dwarfing

resulted from changes in cell expansion and maturation processes. Leaf anatomy was characterized by lack of airspace in the mesophyll, and the palisade layer showed only very limited elongation. Interestingly, growth at 36°C substantially corrected the dwarf phenotype, including reconstitution of clearly defined palisade and spongy mesophyll layers. At high temperatures, the stearate content was still abnormal, suggesting that the consequences of high 18:0 on *fab2* plants may be mediated through an effect on membrane structure. Dwarfing may have resulted from increased saturation of membrane lipids, causing a decrease in membrane fluidity and distorting turgor-driven cell expansion. Temperature increases may have helped to restore fluidity of the membranes, thus normalizing the phenotype.

Overexpression of cytokinins

Overexpression of cytokinins, a class of phytohormones, can have dramatic effects on plant development. Continuous production of cytokinins via fusion of the *Agrobacterium tumefaciens* isopentenyl transferase (*ipt*) gene to a constitutive promoter produced shooty transgenic plants that failed to root (Klee et al., 1987). Medford et al. (1989) placed the *ipt* gene under control of a heat-inducible promoter (maize hsp 70) and used this to transform both tobacco and *Arabidopsis*. In tobacco, *ipt* expression caused release of axillary buds, decreased height, reduced stem and leaf area and an underdeveloped root system. Another

approach utilized the *rolC* gene of *Agrobacterium rhizogenes*, which presumably encodes a cytokinin-ß-glucosidase (Estruch et al., 1991). This enzyme appears to hydrolyze inactive cytokinin glucosides, thus liberating active forms of cytokinins. Transgenic plants expressing this gene were dwarfed with thin and lanceolate leaves, had reduced leaf chlorophyll content, were male sterile, and showed increased root growth *in vitro*. Transgenic variegated plants, in which sectors expressed *rolC*, were produced through use of the *Ac* transposon. *Ac* was placed between the 35S promoter and *rolC* coding sequence; excision produced pale green sectors intermixed with normal dark green tissue. These sectors showed reduced total thickness, irregularly sized and shaped mesophyll cells (typically smaller and flatter), higher cell density, and larger intercellular spaces in the spongy mesophyll.

The Schizoid mutant

Medford et al. (1992) described several *Arabidopsis* mutants, one of which was Schizoid (*Shz*), which forms multiple vegetative shoot apices. *Shz* was identified during a screening of T-DNA tagged mutants. In *Shz*, cells at the base of the meristem, the rib zone, are not in orderly files, and cytoplasm within these cells is abnormally arranged. After 16 d, main stem cells, the interior of which are derived from the rib zone, are necrotic; as are some lateral derivatives of the apical meristem. Upon death of the apex, axillary meristems began to grow. It was suggested that this

necrosis may prevent transmission of an inhibitory signal from the apex (or the signal is not properly made), leading to release of axillary buds and the *Shz* phenotype.

MADS box proteins

Homeotic mutants are mutants with a normal organ in a place where an organ of another type is typically found. Many floral homeotic mutants have been identified. Most floral organ identity genes are regulated at the RNA level and many floral control genes appear to encode transcription factors (Weigel & Meyerowitz, 1994). One such class of transcription factors contains an amino-terminal DNA-binding and dimerization domain (the MADS box) named for the first four members of this family: MCM1, AG, DEF and SRF (Schwarz-Sommer et al., 1990). An additional domain (the K-box) exhibits similarity to the coiled coil domain of keratins and may play a role in protein-protein interactions. Antisense expression of the MADS box-containing *Nicotiana tabacum* AGAMOUS (NAG1) gene in tobacco resulted in petaloid stamens in the flowers (Kempin et al., 1993). Presumably down-regulation of this homeotic gene allowed antagonistic homeotic transcription factors to influence floral morphology.

Homeodomain proteins

Recently, different homeodomain proteins have been overexpressed in tobacco, and interesting pleiotropic alterations in vegetative and/or floral development were observed. Homeodomain genes share a conserved 183-

bp nucleotide sequence known as the homeobox, which encodes the 61 amino acid DNA-binding homeodomain and plays an important role in developmental decisions controlling cell specification and pattern formation (Scott et al., 1989). In *Arabidopsis* these putative transcription factors often also contain leucine zipper dimerization domains, which presumably would allow for the formation of both homo and heterodimers.

Dominant mutations of the *Knotted* locus (*Kn1*) in maize greatly alter leaf morphology, causing protrusions or knots to form along the lateral veins. Knots result from new cell divisions and a change in the plane of growth (Hake et al., 1989). Normally *Kn1* is expressed in apical meristems of vegetative and floral shoots, and is downregulated as leaves and floral organs are initiated (Smith et al., 1992). KNOTTED-1 is a member of a maize homeobox gene family (Vollbrecht et al., 1991). Overexpression of *Kn1* in tobacco caused a switch from determinate to indeterminate cell fates (Sinha et al., 1993). Phenotypes were variable and depended on the level of KN1 protein, but included dwarfing with rumpled or lobed leaves, lack of apical dominance, ectopic shoot formation on leaf surfaces and changes in leaf mesophyll. Leaves were roughly twice as thick as the wild type and showed either a disorganized palisade parenchyma layer, absence of this layer, or large cells with no distinction between palisade and spongy parenchyma.

Transgenic tobacco plants expressing *Athb-1* (a homeobox gene from *Arabidopsis* of unknown function) were deetiolated in the dark and had

lesions in development of the palisade parenchyma layer in the light (Aoyama et al., 1995). Light green sectors were formed in leaves and cotyledons, whereas other organs in the transgenic plants remained normal. Anatomical changes within these sectors included formation of "spongy-like" cells in place of the normal columnar palisade cell layer. It was suggested that replacement of palisade parenchyma cells by spongy mesophyll cells caused the light green coloration, possibly because a palisade parenchyma cell contains more chloroplasts than a spongy mesophyll cell.

*ZmHox1a* and *ZmHox1b*, *Zea mays* homeobox genes, were overexpressed in tobacco (Uberlacker et al., 1996). Transgenic plants showed a variety of phenotypes including size reduction, leaf narrowing, release of axillary buds and homeotic floral transformations. Floral changes included formation of petaloid stamens. Leaves have not been sectioned to determine if any changes in the palisade layer have occurred (W. Werr, personal communication).

MADS/homeodomain interactions

Grueneberg et al. (1992) studied interactions between a homeodomain protein (Phox1) and a MADS transcription factor, the serum response factor (SRF). DNA mobility shift assays demonstrated specific interactions between these two transcription factors. It was suggested that homeodomain proteins might determine where SRF-containing

complexes (or complexes anchored by other MADS box proteins) are assembled in the genome. In this way, homeodomain proteins might help establish cell identity by determining which genes are activated in response to an otherwise generic inductive signal. Thus, as a cell undergoes a developmental switch and produces a new homeodomain protein, MADS box proteins would be recruited to new sites. This would then alter the cell's response to subsequent signals (Grueneberg et al., 1992).

# CHAPTER II

## EXPRESSION OF YEAST *FRE1* AND *FRE2* GENES IN TOBACCO


### Abstract

Two yeast Fe(III) reductase genes, *FRE1* (Dancis et al., 1992) and *FRE2* (Georgatsou and Alexandraki, 1994), were introduced into tobacco via *Agrobacterium*-mediated transformation. Fe(III) reductase activity was measured in homozygous transformants containing *FRE1*, *FRE2*, or both. Homozygous lines containing only *FRE1* or *FRE2* differed in Fe(III) reduction, and some lines had higher Fe(III) reduction than the control. The highest Fe(III) reduction levels were found in lines containing both *FRE1* and *FRE2*. Results from liquid reductase assays suggested three to four times more Fe(III) reduction in these transformants as compared to controls, and visual plate assays showed reduction along the entire length of the roots. One *FRE1*-containing line initially exhibited chlorosis on medium with low Fe at pH 7.5, but was later able to resume normal growth. Other transformants and the control remained chlorotic.


### Introduction

Since Fe is an essential micronutrient required for normal functioning of plant cells, Fe uptake and storage are highly regulated processes. Free Fe(II) ions are readily taken up by roots (Chaney et al., 1972), but soil often contains insoluble Fe(III) oxides and hydroxides. One key mechanism

utilized by plants to make Fe more available for absorption is the reduction of Fe(III) to Fe(II) via an inducible, PM-bound Fe(III) reductase (Bruggemann et al., 1990).

One strategy to increase Fe(III) reduction may be to manipulate Fe(III) reductases through genetic engineering. In recent years, several Fe(III) reductases have been isolated using techniques that allow isolation of PMs without contaminants (Larsson and Moller, 1990). This has aided in kinetic and biochemical characterization of plant Fe (III) reductases (Moog and Bruggemann, 1994). Fe (III) chelate reductases have been partially purified from PMs of Fe-deficient tomato roots using affinity chromatography (Holden et al., 1994). This led to identification of a 35 kDa polypeptide which is presumably the catalytic component of a multi-subunit Fe (III) chelate reductase. However, plant genes encoding such reductases have not yet been isolated.

Two Fe(III) reductase genes have been isolated from the yeast *Saccharomyces cerevisiae*. Dancis et al. (1992) identified *FRE1* via complementation of a mutant yeast lacking externally directed PM reductase activity. A second Fe(III) reductase gene, *FRE2*, was identified during sequencing of yeast chromosome XI (Georgatsou and Alexandraki, 1994). The combination of FRE1 and FRE2 was shown to account for the total membrane-associated Fe(III) reductase activity in *S. cerevisiae*. Even though *FRE1* and *FRE2* encode enzymes with the similar functions, they do not show significant similarity at the nucleotide level and only have

borderline similarity in deduced amino acid sequence. This suggests that the two genes may have been derived from a common ancestral gene following extensive nucleotide divergence. The only preserved sequences are most likely those ensuring enzymatic function and membrane localization.

Some yeast genes have been successfully expressed in plants (Colau et al., 1987; von Schaewen et al., 1990). Therefore, incorporation of the *FRE* genes in plants may also lead to formation of functional proteins and, consequently, enhancement of Fe(III) reduction. This, in turn, may result in a decrease in Fe chlorosis in transgenic plants. Here I report on the genetic transformation of *Nicotiana tabacum* with *FRE1* and *FRE2* and characterization of transformed plants with regard to Fe(III) reductase activity.

## Materials and Methods

### PCR of *FRE1*

The following primers were used to amplify *FRE1* from high molecular weight *S. cerevisiae* genomic DNA (obtained from Dr. S. Johnston):

FRE1 5′ (5′ CCGGGGATCCATGGTTAGAACCCGTGTATTATTCTGC 3′)

FRE1 3′ (5′ TTATGAATTCGGGGCCTTACCATGTAAAACTTTCTTC 3′)

PCR was performed with 40 cycles of 92°C, 2 min.; 52°C, 2 min.; 72°C, 3 min. Both *Bam*HI and *Nco*I sites were engineered into the 5′ primer and an *Eco*RI site was built into the 3′ primer to facilitate future cloning.

### Cloning of *FRE1* into Plasmids

The PCR product (2.1 kB) was blunt-ended using T4 DNA polymerase and treated with T4 polynucleotide kinase. After gel purification, this fragment was ligated to dephosphorylated *Sma*I-cut pUC18 (Vieira and Messing, 1982; Yanisch-Perron et al., 1985). *E. coli* DH5$\alpha$ competent cells were transformed with this construct using the suggested protocol (Gibco BRL, Inc.). Ampicillin-resistant transformants with *FRE1* in the reverse orientation (to gain necessary 3′ *Bam*HI and *Hind*III restriction sites from the polylinker) were identified and the plasmid was designated pUC18F. A Qiagen Midiprep procedure was used to purify pUC18F plasmid according to the suggested protocol (Qiagen, Inc.). This plasmid was cut with *Nco*I and *Hind*III to obtain an insert lacking the 5′

*Bam*HI site and ligated to pTL27N, a protein-expression plasmid, cut with the same enzymes (Carrington et al., 1987). Competent DH5*α* cells were transformed with this plasmid and ampicillin resistant colonies were selected. A plasmid comprised of pTL27N with the 2.1 kB insert (pTL27NFRE1) was purified by the Qiagen procedure, cut with *Nco*I and *Hind*III, and the *FRE1*-containing fragment cloned into pTC:MT-1. pTC:MT-1 is identical to pTL27N, except for the presence of a 5' *Bam*HI site upstream of the *Nco*I site and the Kozak sequence (Kozak, 1986) between *Bam*HI and *Nco*I. The *Bam*HI cassette (now with the added 5'Kozak sequence for eukaryotic translation) was cut from pTC:MT-1 and ligated to *Bam*HI-cut dephosphorylated pPEV plasmid (Lindbo and Dougherty, 1992). pPEV is a binary vector that can be maintained in both *E. coli* and *A. tumefaciens* and contains an enhanced CaMV 35S promoter driving the inserted gene and the *nptII* gene conferring kanamycin resistance in plants. *E. coli* TG-1 competent cells were transformed with this vector and kanamycin-resistant colonies selected. Minipreps were performed and bacteria with *FRE1* cloned in both orientations in the plasmid vector were selected. All bacterial stocks were stored in 15% glycerol at -80°C.

**PCR of *FRE2***

The following primers (with *Bam*HI sites engineered into both) were used to amplify *FRE2* from high molecular weight *S. cerevisiae* genomic DNA:

FRE2 5′ (5′ CCAACGGGATCCATGCATTGGACGTCCATCTTGAGCGC 3′)

FRE2 3′ (5′ AAGTGGATCCTGATCACCAGCATTGATACTCTTCAAAG 3′)

PCR was performed with two cycles of 94°C, 1 min; 37°C, 2 min.; 42°C, 2 min.; 55°C, 2 min.; 72°C, 2 min.; then 40 cycles of 92°C, 2 min.; 60°C, 2 min.; 72°C, 3 min.

**Cloning of *FRE2* into Plasmids**

The PCR product was cut with *Bam*HI and cloned into *Bam*HI-cut dephosphorylated pUC18. pUC18FRE2 plasmid, purified by the Qiagen procedure, was cut with *Bam*HI and the resulting 2.1 kb fragment cloned into dephosphorylated *Bam*HI-cut pPEV plasmid (producing pPEVFRE2). The binary plasmid pGPTV-BAR (Becker et al., 1992), conferring bialaphos resistance, was cut with *Xba*I and *Eco*RI, as was pPEVFRE2. The resulting *FRE2* cassette from pPEVFRE2, containing the enhanced 35S promoter upstream of *FRE2*, was ligated to pGPTV-BAR. This construct allowed plant selection using the herbicide bialaphos and bacterial selection using kanamycin. The plasmid was transformed into competent *E. coli* DH5$\alpha$.

**Sequencing of *FRE1***

Sequencing was performed by the Oregon State University Central Services laboratory on Applied Biosystems sequencers (models 373A and 377). Both pTL27NFRE1 and pUC18F were used as templates.

***In Vitro* Transcription/Translation of *FRE1***

*Hind*III-linearized pTL27NFRE1 was used as a template for transcription off the T7 promoter and subsequent translation in a TNT rabbit reticulocyte lysate system (Promega). The manufacturer's protocol was followed, using 2 $\mu$l of $^{35}$S-methionine (NEN Dupont) and 2.5 $\mu$l of canine microsomes (Boehringer-Mannheim) when microsomes were included in the reaction. After completion of the reaction, 5 $\mu$l was added to 20 $\mu$l of SDS reducing buffer, boiled, and fractionated on a SDS minigel (Bio Rad). The gel was placed onto filter paper (Bio Rad # 1650962), dried under vacuum in a slab dryer (Bio Rad #443), and bands were visualized using autoradiography film (Hyperfilm-MP, Amersham).

**Transformation of *Agrobacterium***

The protocol of Hofgen and Willmitzer (1988) was used to transform *A. tumefaciens* EHA 105. 500 $\mu$l of an overnight culture of EHA 105 in YEP medium (10 g/L Bacto peptone, 10 g/L Bacto Yeast Extract, 5 g/L NaCl) was used to inoculate 10 ml of YEP and shaken vigorously at 28°C for 3-4 h. After centrifugation at 4°C (20 min., 3000xg), the pellet was resuspended in 1 ml of cold TE buffer (10 mM Tris plus 1 mM EDTA, pH

7.5). Cells were pelleted in a microcentrifuge at 4°C and resuspended in 1 ml of YEP, and 500 μl was placed into each of two 2 ml cryovials (Corning) on ice. Plasmid DNA (1 μg) was added to 500 μl of the bacteria, mixed well, and incubated on ice for 5 min. The cryovials were placed into liquid nitrogen for 5 min., then held in a 37°C water bath for 5 min., after which 1 ml of YEP medium was added and the vials were shaken at 28°C for 2-4 h. Dilutions of this were then spread onto YEP-kanamycin (50 μg/ml) plates and incubated for 2-3 days at 28°C. Individual colonies were re-streaked onto YEP-kanamycin plates and *FRE1*- or *FRE2*- specific primers were used for PCR analysis. PCR-positive colonies were grown in liquid YEP-kanamycin and stored in 15% glycerol at -80°C.

**Transformation of Tobacco**

*A. tumefaciens* EHA105 containing *FRE1* or *FRE2* was grown in 10 ml YEP-kanamycin (50 μg/ml) overnight at 28°C. After centrifugation in sterile polypropylene tubes (Falcon #2059) at room temperature (15 min, 2500 rpm), the pellet was resuspended in 10 ml SIM medium (20 g/L sucrose, 20 mM sodium citrate, pH 5.2) with 100 μM acetosyringone (3′,5′-dimethoxy-4′-hydroxy-acetophenone, Aldrich). The cultures were incubated for 5 h at 28°C, then diluted 1:10 in Murashige and Skoog (MS) medium without hormones (Murashige and Skoog, 1962). Leaf discs were incubated in this solution for 10 min., blotted dry on sterile filter paper and placed onto regeneration medium (MS with 1 mg/L BAP and 0.1 mg/L NAA)

containing 100 μM acetosyringone. After 48 h, leaf discs were dipped in 400 mg/L timentin (SmithKline Beecham), blotted and placed onto fresh regeneration medium with 400 mg/L timentin and either 400 mg/L kanamycin (Sigma) for transformation with *FRE1*, or 5 mg/L bialaphos (Meiji Seika Kaishya, Ltd.) for transformation with *FRE2*. Once shoots developed, they were excised and placed onto MS medium without growth regulators, with 400 mg/L timentin + 400 mg/L kanamycin (or 10 mg/L bialaphos) for rooting. Larger rooted plants were then transferred to a mist bench and eventually to greenhouse benches.

**Scoring of Transgenic Progeny**

Seeds were wrapped in small Miracloth (Calbiochem) bags, dipped in 70% ethanol for 30 sec., left in 20% Clorox with 0.1% (v/v) Tween 20 detergent for 20 min., and rinsed twice with sterile distilled water. Seeds were placed onto MS medium with either 400 mg/L kanamycin or 10 mg/L bialaphos. Seedlings were visually scored for resistance or susceptibility to the selective agents. Resistant plants were grown in the greenhouse and selfed progenies were screened again to identify homozygous lines.

**Southern and Northern Blotting**

Genomic DNA was extracted from 2 g tobacco leaf tissue using a CTAB (hexadecyltrimethylammonium bromide) protocol (Doyle and Doyle, 1990). Polysaccharides were removed (Murray and Thompson, 1980) and 10 μg DNA was digested with *Eco*RV. After DNA separation on a 1%

agarose gel, the gel was treated with 0.25 M HCl, with 0.5 M NaOH plus 1.0 M NaCl for denaturation of DNA, neutralized with 0.5 M Tris-HCl pH 7.4 plus 3.0 M NaCl, and blotted onto a nylon Zeta-Probe (Bio Rad) membrane. After washing briefly in 2x SSC, the membrane was air dried and heated at 80°C for 1 h. The $\alpha$-$^{32}$P dCTP-labelled probe, covering the entire *FRE1* or *FRE2* gene, was synthesized using Ready-To-Go DNA labelling beads (Pharmacia) following the manufacturer's protocol and purified on a Biospin 6 chromatography column (Bio Rad). The membrane was pre-hybridized for 30 min. and hybridized for 24 h in 0.25 M $Na_2HPO_4$, pH 7.2, plus 7% SDS (sodium dodecyl sulfate) according to the Zeta-Probe protocol. A Robbins Model 1000 hybridization incubator set at 50°C was utilized for both prehybridization and hybridization procedures. The membrane was washed five times with 2x SSC + 0.1% SDS (50°C) and two times with 1x SSC + 0.1% SDS (60°C). Autoradiography was performed using Hyperfilm-MP (Amersham).

For Northern blotting, mRNA was isolated from 0.8 g leaf tissue according to the Poly-A-Tract System 1000 protocol (Promega). Total RNA was isolated from 0.1 g leaf tissue according to the TRIzol reagent protocol (Gibco BRL). PolyA RNA (1 $\mu$g) or total RNA (15 $\mu$g) was separated on a formaldehyde gel (Davis et al., 1986), the suggested protocol (Bio Rad) was used for capillary transfer to Zeta-Probe membrane, and hybridization was performed in the same way as for the Southern blot (see above). For membrane stripping, in order to re-probe, the Bio Rad protocol was followed.

**Fe Reductase Assay**

Fe (III) reduction was examined quantitatively using a liquid assay and qualitatively visualized on agarose plates. Tobacco seedlings were grown in a 25°C culture room ( 16 h light, 10 $\mu$mol.m$^{-2}$.s$^{-1}$) on half-strength MS medium containing 10 $\mu$M Fe (II) EDTA and 6 g/L Bacto agar (pH 5.7) in 100 x 15 mm square petri dishes. Dishes were positioned vertically to allow roots to grow downward along the surface of the medium. After 2 or 3 weeks, seedlings were removed and floated in ~200 ml sterile distilled water for at least 3 min. before reductase assays.

Root Fe(III) reduction was determined spectrophotometrically using the reagent BPDS (bathophenanthrolinedisulfonic acid). This chemical chelator forms a red colored complex with Fe(II) ($[Fe^{+2}(BPDS)_3]^{-4}$) but not with Fe(III). Molar absorption of this complex is 22140 $M^{-1}cm^{-1}$ at 535 nm (Kojima and Bates, 1981). Since $Co^{+2}$, $Mn^{+2}$, $Zn^{+2}$ or $Cu^{+2}$ interfere with Fe(III) reduction, they were omitted from the assay medium (Romheld and Marschner, 1983). For assays, MS medium (pH 6.0) was used, but Fe or the interfering metals were omitted and 0.1 mM Fe(III) EDTA and 0.3 mM BPDS were added after autoclaving (Marschner et al., 1982). Four roots were placed into each vial with 4 ml sterile assay medium. Manipulations were carried out in the dark to prevent photoreduction of Fe(III). Vials were sealed in aluminum foil and placed on a slow-moving orbital shaker overnight. After 24 h, absorbance was determined with a dual beam

spectrophotometer (Beckman, model 34) and Fe reduction calculated from the absorption at 535 nm minus the blank (containing no tissue). Roots were blotted dry, weighed, and Fe reduction expressed as nmol/mg fresh weight over 24 h.

For embedding in agar, assay medium (see above) containing 0.25% agarose (Sigma A-6013) was distributed into petri plates. Seedlings were placed onto this medium and roots were pressed gently into the agarose. Plates were sealed with parafilm and placed in the dark at 25°C. Roots were observed periodically over a 24 h time period.

**Response to Fe-Deficient Medium**

Two different media were used to test whether presence of the *FRE* genes could overcome Fe-deficiency chlorosis. Initially, MS-0 medium without Fe but with 1.0 mM potassium bicarbonate and 6 g/L Difco Bacto agar (providing a low concentration of Fe to the medium) was used. The pH of the medium was 7.5. Nine-day-old seedlings were transferred onto 50 ml of this medium in Magenta boxes (five seedlings per box). In later studies, most of the nutrients (except N, P and K) and the DTPA (diethylenetriaminepentaacetic acid, Aldrich) of the solution devised by Chaney et al. (1992) were included in the medium, in addition to 1.0 mM potassium bicarbonate and 0, 0.01, 0.1 or 10 $\mu$M FeDTPA. Agarose (0.25%) was used as the gelling agent. Disinfested seeds were sown directly on this medium in Magenta boxes (four seedlings per box). The

advantage of the latter medium is the presence of high levels of DTPA chelator, which ensures the availability of Mn, Mg, Fe, Cu, and Zn at high pH.

Spectrophotometric chlorophyll determination in DMF (N,N-dimethyl formamide) was as described (Moran, 1982; Dolcet-Sanjuan et al., 1992). Absorption of DMF-solubilized chlorophyll extracts (at 647 nm and 664.5 nm) was recorded. Chlorophyll amount ($\mu$g/ml) was determined using the equation 17.90 $A_{647}$ + 8.08 $A_{664.5}$ (Inskeep and Bloom, 1985).

## Results

### *In Vitro* Transcription/Translation

Sequencing of the *FRE1* PCR product indicated that it was correct. Attempts to express full-length FRE1 protein in *E. coli* resulted in death of the bacterium upon IPTG induction. This is expected, since overexpression of eukaryotic membrane proteins in prokaryotes often disrupts normal membrane functions (Schertler, 1992). In order to demonstrate that a protein of correct size could be produced from the *FRE1* PCR product, *in vitro* transcription/translation was performed. Since several potential glycosylation sites were identified in FRE1 (Dancis et al., 1992), canine pancreatic microsomes were also included in the transcription/translation reaction. Microsomes are endoplasmic reticulum vesicles which support cotranslational processing of proteins (i.e. signal peptide cleavage, membrane insertion, translocation, and glycosylation), provided translation

products contain the necessary information. Glycosylation results in an increase in molecular weight. Translation in the presence of $^{35}$S-methionine produced a protein of the correct size (Fig. 2.1, lane 1). The addition of canine microsomes brought about an increase in molecular weight most likely as the result of glycosylation (Fig. 2.1, lane 2). A second band of unknown origin occurred beneath the FRE1 product, but did not change position with canine microsomes.

**Tobacco Transformants**

Twenty one independent *FRE1* transformants were generated. As expected, progenies ($S_1$) from primary transformants segregated for kanamycin resistance. Some progenies fit 3:1 ($kan^r$:$kan^s$) segregation ratios; others had higher proportions of $kan^r$ plants possibly due to multiple insertions. Plants from three independent families were selfed to generate lines uniformly homozygous for kanamycin resistance (and the presence of *FRE1*). These were designated FRE1-A, FRE1-B and FRE1-C. Eighteen independent *FRE2* transformants were generated. Plants from three $S_1$ lines were selfed to give rise to lines homozygous for bialaphos resistance, FRE2-A, FRE2-B and FRE2-C. FRE1-C was utilized for re-transformation with FRE2, and sixteen independent $S_1$ lines were generated. One $S_1$ line was selfed to homozygosity (FRE1 + 2-A). In addition, three homozygous kanamycin-resistant no-insert (NI) control lines were generated, and NI-A was used as a control for all experiments.

**Fig. 2.1.** *In vitro* transcription/translation of *FRE1* without canine microsomes (lane 1) and with microsomes (lane 2).

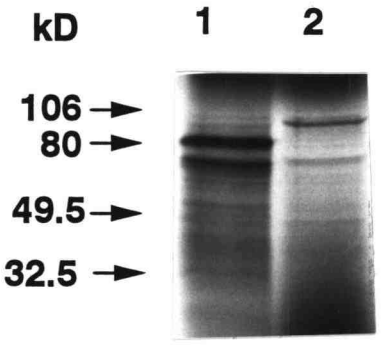



Figure 2.1.

Antisense transformants (with *FRE1* and *FRE2* in the reverse orientation relative to the CaMV 35S promoter) were also produced, withthe idea that these may suppress endogenous Fe(III) reductases if there was sufficient homology. Transgenic plants containing *FRE1* or *FRE2*, in sense or antisense orientations, did not differ visibly from control plants.

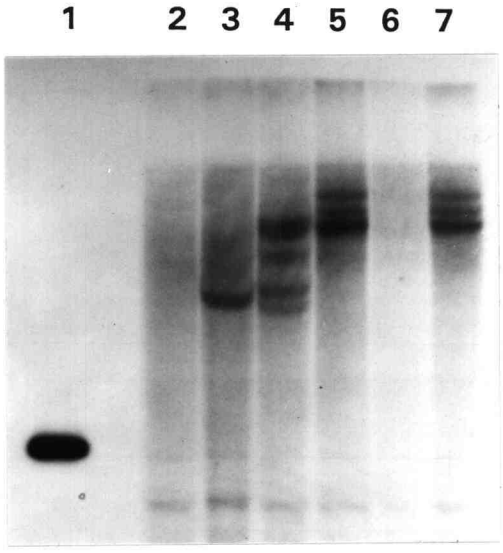
**Southern and Northern Analyses**

To determine the number of *FRE1* and *FRE2* inserts in the tobacco transformants, Southern blots of *Eco*RV digests were probed with *FRE1* (Fig. 2.2 A) and *FRE2* (Fig. 2.2 B). *FRE1* does not contain any *Eco*RV sites, whereas *FRE2* and the CaMV promoter both contain one *Eco*RV site. Four clear bands were visible in FRE1-A, one in FRE1-B, and three in FRE1-C when *FRE1* was used as a probe (Fig. 2.2 A). As expected, FRE1 + 2-A, a derivative of FRE1-C, also had three bands. An additional low MW band was present in all lines, including NI-A, indicating there may be a gene with some homology to *FRE1* in tobacco. However, homology may be low since the band is faint and low-stringency conditions were used for hybridization.

In blots developed with *FRE2* as probe, several bands were visible in lanes corresponding to the transformed lines (Fig. 2.2 B). However, since *FRE2* and the promoter region contain an *Eco*RV site, the band common to all transformants was the result of excision of the fragment between these sites. Not counting this band, the three *FRE2* lines have one, one, and two bands respectively, and the FRE1 + 2-A line two *FRE2* inserts.

**Fig. 2.2.** Southern blots of *Eco*RV-digested tobacco genomic DNA. (A) *FRE1* probe. Lane 1: 50 pg *FRE1* control; lane 2: NI-A; lane 3: FRE1-B; lane 4: FRE1-A; lane 5: FRE1-C; lane 6: FRE2-A; lane 7: FRE1 + 2-A. (B) *FRE2* probe. Lane 1: 50 pg *FRE2* control; lane 2: NI-A; lane 3: FRE1 + 2-A; lane 4: FRE2-A; lane 5: FRE2-C; lane 6: FRE2-B.

A




B

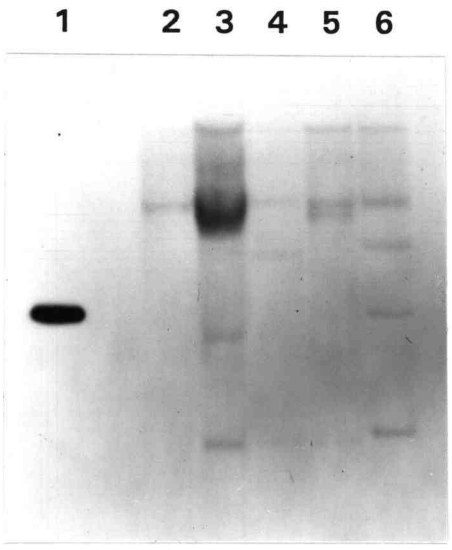



**Figure 2.2.**

Again, one non-specific band is present, but this fragment is much larger than the one recognized by *FRE1*, indicating that the *FRE1* and *FRE2* probes hybridize to different plant genes. Presence ofthe entire *FRE2* gene in each transformant was demonstrated by PCR using the border regions as primers (Fig. 2.3).

Verification that mRNA was produced came from Northern blot analysis. Blots probed separately with *FRE1* and *FRE2* had a single non-specific band common to all lanes (Fig. 2.4 A and B). The origin of this band is not clear; it may indicate the presence of a tobacco gene with some homology to these genes or it could be due to other nonspecific recognition. Two additional bands occurred in the FRE1-B, FRE1-C and FRE1 + 2 lanes. The upper band represents the complete transcript, while the low molecular weight band may be due to an internal transcription site. Probing with *FRE2* showed only one additional band in FRE2-A and FRE1 + 2-A. These Northern blots show quite clearly that message is made and is correctly detected by the two different probes in transformed plants.

**Fe(III) Reduction**

Liquid Fe(III) reductase assays were performed using excised roots from seedlings grown on Fe-containing medium (Fig. 2.5). *FRE1*-containing plants showed variable reductase activity, ranging from slightly more than the NI-A control (FRE1-A), to a modest increase (FRE1-C) with a large standard error.

**Fig. 2.3.** PCR using *FRE2* border primers. Lane 1: markers; lane 2: *FRE2* (+) control; lane 3: no template control; lane 4: NI-A; lane 5: FRE1 + 2-A; lane 6: FRE2-A; lane 7: FRE2-B; lane 8: FRE2-C.

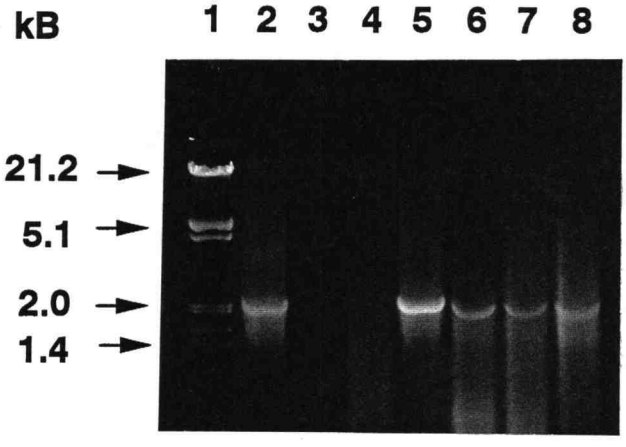



Figure 2.3

**Fig. 2.4.** Northern blots. (A) *FRE1* probe, (B) *FRE2* probe. Lane 1: 40 pg *FRE1* DNA; lane 2: 40 pg *FRE2* DNA; lane 3: 1 $\mu$g NI-A mRNA; lane 4: 1 $\mu$g FRE1-B mRNA; lane 5: 1 $\mu$g FRE2-A mRNA; lane 6: 15 $\mu$g FRE1 + 2-A total RNA; lane 7: 1 $\mu$g FRE1-C mRNA.

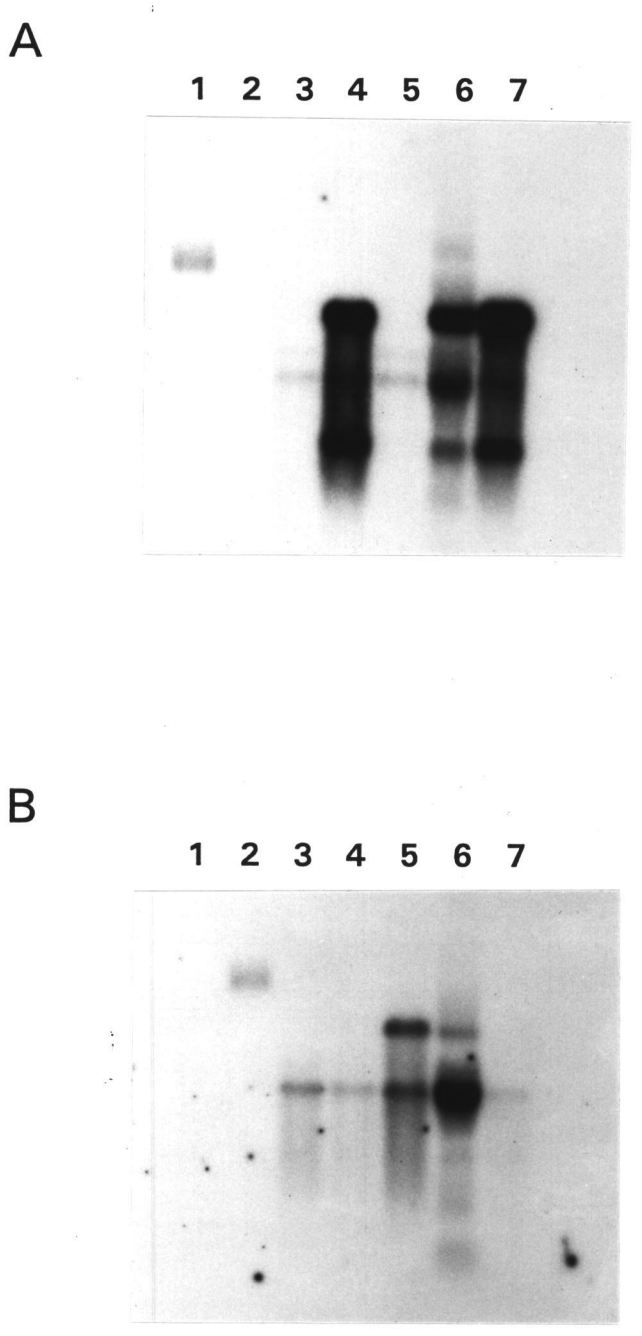



**Figure 2.4.**

**Fig. 2.5.** Root Fe(III) reduction in lines with *FRE1*, *FRE2* and *FRE1+2*. Vertical bar indicates standard error of the mean.

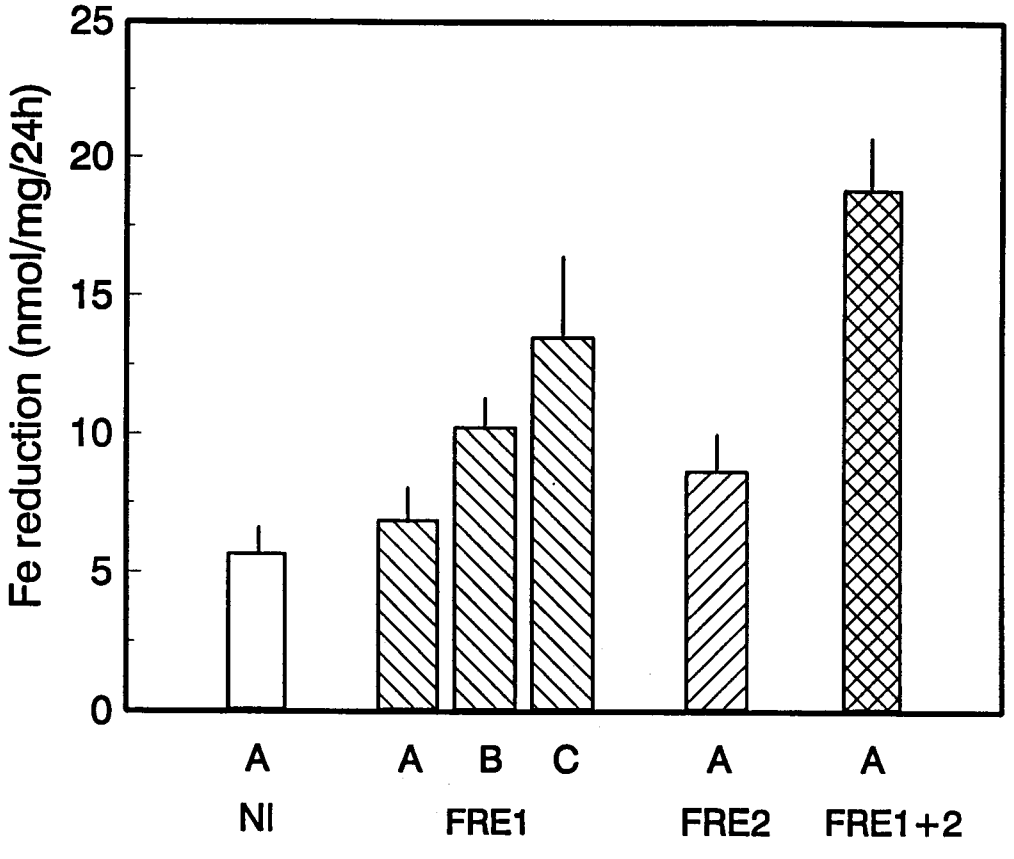

25
20
15
10
5
0
Fe reduction (nmol/mg/24h)
A
A
B
C
A
A
NI
FRE1
FRE2
FRE1+2

Figure 2.5.

However, Yamaguchi et al. (1995) also transformed tobacco with *FRE1*, and reported no significant increase in Fe(III) reduction. The one line transformed with *FRE2* showed only a slight increase in Fe(III) reductase activity (FRE2-A). When FRE1-C was re-transformed with *FRE2*, three to four times more Fe(III) reduction was obtained (FRE1 + 2-A). Also,in seedlings of additional lines that contain *FRE1* and segregate for *FRE2* (but had been grown on bialaphos-containing medium to select for the presence of *FRE2*), Fe(III) reductase activity was much higher than in the control (Fig. 2.6) and similar to that of the homozygous line.

The high Fe(III) reductase activity in FRE1 + 2-A was confirmed by assays with seedlings embedded in semi-solid medium (Fig. 2.7). As expected, reductase activity could be seen at the tip of most roots, typical for endogenous plant Fe(III) reductase. However, FRE1 + 2-A roots showed reduction along their entire length, which could signify expression of genes driven by the constitutive CaMV 35S promoter.

**Growth Responses to Fe-Deficient Medium**

On medium without or with low (0.01, 0.1 or 1 $\mu$M) Fe, seedlings of lines containing *FRE1* or *FRE2* became chlorotic and growth was inhibited relative to the Fe concentration. No differences in chlorophyll concentrations could be detected between these lines and the NI-A control. However, an interesting phenotype was identified among progeny of one transgenic line containing *FRE1*, FRE1-D.

**Fig. 2.6.** Root Fe(III) reduction in lines containing *FRE1+2*. Vertical bar indicates standard error of the mean.

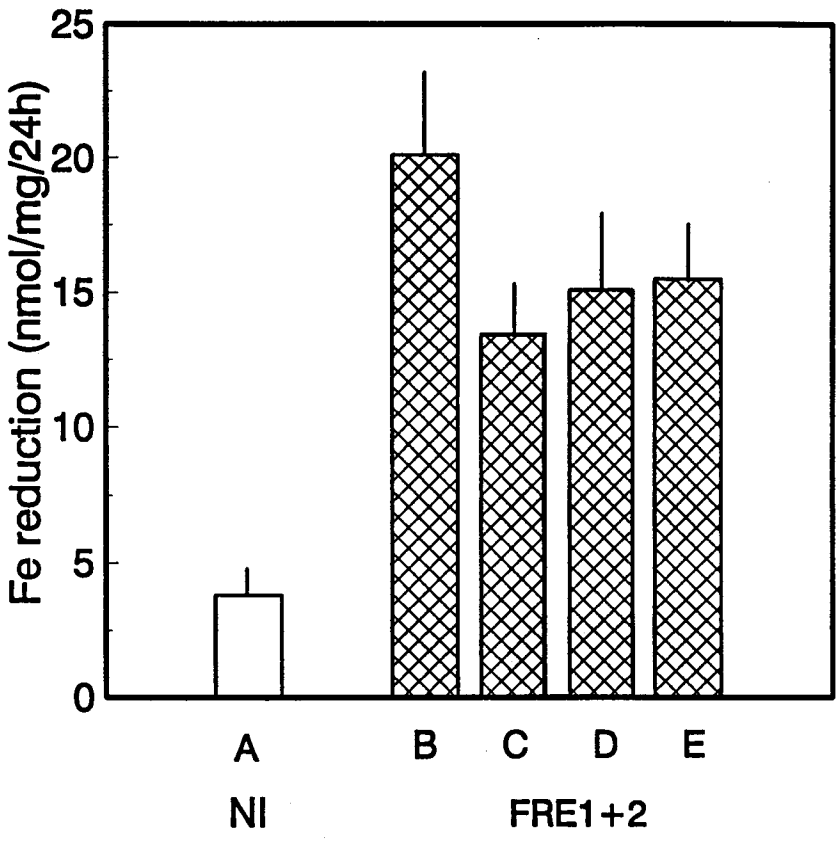
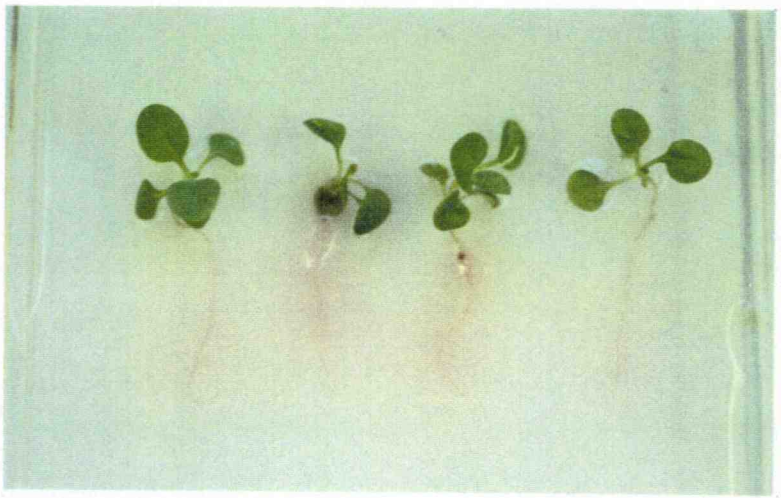



Figure 2.6.

**Fig. 2.7.** Fe(III) reduction visualized by embedding seedlings in medium containing Fe(III) and BPDS for 6 h. Top: NI-A, 4-week-old seedlings; Middle: FRE1 + 2-A, 4-week-old seedlings; Bottom: FRE1 + 2-A, 5-week-old seedling.



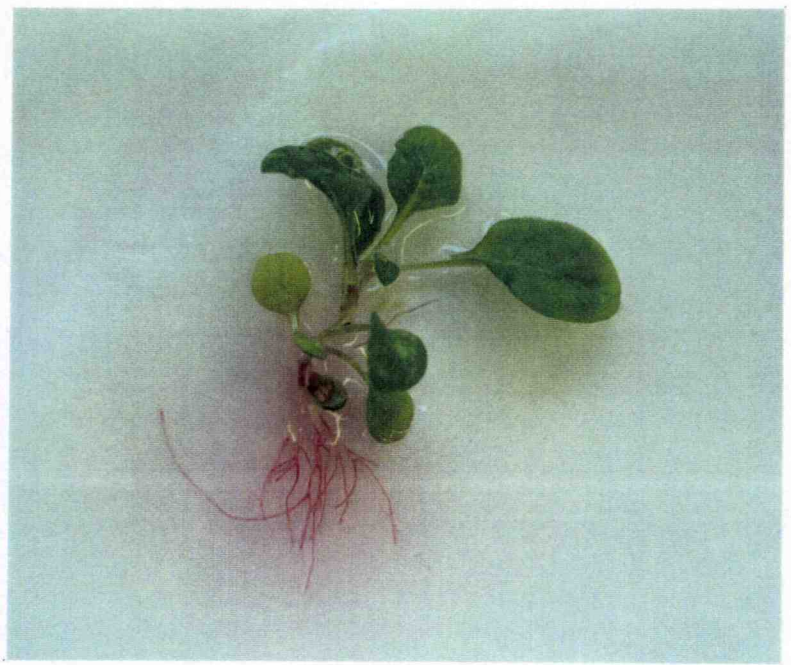


**Fig. 2.8.** Tobacco seedlings grown on high pH, low Fe medium for 8 weeks. NI-A (left), FRE1-D (right).

Figure 2.8.

Within two weeks on the high pH MS-0 medium, seedlings of this line, as well as all other transformed and control tobacco lines, became highly chlorotic and stopped developing new leaves. After four weeks, new leaves appeared on FRE1-D plants and eventually plants fully recovered (Fig. 2.8). Chlorophyll determination forFRE1 + 2 transformants has not yet been performed, but will be examined in the near future.

## Discussion

The finding that the incorporation of yeast reductase genes in tobacco can enhance root Fe(III) reduction is quite interesting. The much higher reductase activity along the roots of FRE1 + 2, as compared to the singly (*FRE1* or *FRE2*) transformed roots, may suggest that there is an interaction between the yeast FRE1 and FRE2 proteins for optimal enzyme function in plants. However, expression of transgenes depends on copy number and position; therefore, it is possible that the higher Fe(III) reduction of the FRE1 + 2 line reflects only the contribution of FRE2. Analysis of additional FRE2 and FRE1 + 2 lines may elucidate the exact contribution of each of the genes.

No differences in chlorophyll content for *FRE1*, *FRE2* and control lines were seen. However, the recent FRE1 + 2 line has not yet been analyzed and thus it is not known whether the combination of FRE1 and FRE2 can produce a marked decrease in Fe chlorosis. It is possible that

increases in additional Fe related proteins, such as the Fe(II) transporter (Eide et al., 1996) are also required for Fe uptake.

Southern and Northern blots show endogenous hybridizing bands in tobacco. It is interesting that *FRE1* probing of *Eco*RV-digested genomic DNA produces a common band of one molecular weight, while *FRE2* probing shows another shared band of higher molecular weight. This could indicate the presence of similar systems in plants. However, hybridization is weak and was carried out at low stringency. Yi et al. (1994) have cloned plant homologs to *FRE1* by probing lambda libraries of *Arabidopsis* genomic DNA. Three different clones with homology to *FRE1* have been identified. It is not yet known whether these clones represent plant Fe(III) reductases.

FRE1-D, which eventually recovers on high pH, Fe-deficient medium, will be studied in more detail. Initial observations suggest that this line may acidify the medium more effectively than controls and other lines. It is not known whether this phenotype is related to the presence of one or more of the three *FRE1* inserts in this line. However, it is clear that the recovery is not due to the action of *FRE1* itself, since none of the other *FRE1*-containing lines displayed this response.

Since the yeast genes are constitutively expressed (due to the CaMV 35S promoter), uniform Fe(III) reductase activity can be expected; perhaps even in plant parts other than the roots. Visual assays of FRE1 + 2 roots showed Fe(III) reduction in areas outside the usual localized zones.

Therefore, it may be useful to also examine Fe(III) reductase activity in stems and leaves of transgenic plants. Eventually, Fe-regulated and root-specific promoters may be used in conjunction with the yeast genes. Even though enhanced Fe(III) reductase activity is measured, it is not known whether the enzymes are properly targeted to the PM within the plant cell. Future analyses using antibodies to FRE1 and FRE2 may allow precise cytolocalization of the proteins.

In conclusion, this work presents the first evidence that co-expressed FRE1 and FRE2 may increase Fe(III) reductase activity in plants. This finding provides new research opportunities and raises several interesting questions. Does increased Fe(III) reduction alone enhance the overall Fe metabolism of plants? Will crosses between *FRE1* and *FRE2* homozygotes produce progeny with enhanced Fe(III) reduction, and will transgene dosage be a critical component? What happens to reductase activity in FRE1 + 2 transformants if root tips are excised (eliminating endogenous contributions)? Overexpression of non-plant-derived Fe(III) reductases might be more useful than over-expressing a plant Fe(III) reductase (once cloned), due to the possibility of cosuppression of endogenous plant genes (Dougherty and Parks, 1995). In transgenic plants of the future, yeast-derived genes may serve to supplement the resident plant Fe uptake system, thereby decreasing Fe chlorosis and improving crop yield.

# CHAPTER III

## A TEMPERATURE-DEPENDENT MORPHOLOGICAL MUTANT OF TOBACCO


### Abstract

A recessive temperature dependent shooty mutant (*tds*) of *Nicotiana tabacum* (W38) is described. The mutant phenotype is expressed at low temperature (21°C). Mutant characteristics include formation of thick, narrow leaves with abnormal mesophyll cells and near absence of apical dominance. Most plants remain vegetative, and the few flowers that are formed have petaloid stamens. High temperature (30°C) reverses the mutant phenotype, with formation of normal leaves and restoration of apical dominance. However, many flowers still have petaloid stamens. Reciprocal grafting and auxin-cytokinin interaction experiments with cultured tissues do not suggest shifts in hormone balance. The fatty acid profile appears to be normal. This mutant shares several phenotypic characteristics with transgenic tobacco plants overexpressing maize and *Arabidopsis* homeodomain proteins.

## Introduction

Many mutant phenotypes have been identified among progeny of *Arabidopsis* plants used for *Agrobacterium*-mediated transformation (Feldmann, 1991). A number of interesting phenotypes have been directly attributed to T-DNA tagged genes (Marks and Feldmann, 1989; Yanofsky et al., 1990; Feldmann 1991; Deng et al., 1992; Kieber et al., 1993). Castle et al. (1993) concluded that the transformation process itself may generate mutations not associated with stable T-DNA integration. Mutants, tagged or not, have been useful in the study of plant development (Marx, 1983; Meinke, 1995; Yanofsky, 1995).

Temperature-sensitive mutants have been isolated in plants, and the most abundant category are those showing normal phenotypes at low temperature and mutant phenotypes at high temperature. Some examples include carrot variants unable to complete embryo development (Schnall et al., 1991), an auxin-auxotrophic mutant of *Nicotiana plumbaginifolia* (Fracheboud and King, 1991), and *Arabidopsis* mutants defective in the redifferentiation of shoots from root explants (Yasutani et al., 1994). There are rare cases of high temperatures being permissive and low temperatures restrictive. Two examples are sweetclover mutants defective in chlorophyll production (Bevins et al., 1993) and the *Arabidopsis* mutant, *fab2*, which overproduces the fatty acid stearate (Lightner et al., 1994).

A temperature-dependent pleiotropic mutant was found among the progeny of tobacco transformed by *Agrobacterium*. The binary vector

plasmid was pPEV (Lindbo and Dougherty, 1992) and the T-DNA contained the *nptII* gene plus the yeast ferric reductase gene, *FRE1* (Dancis et al., 1992), driven by an enhanced CaMV 35S promoter. At low temperatures this recessive mutant is characterized by loss of apical dominance, multiple vegetative shoots with thick, narrow leaves, abnormal mesophyll cells, and an occasional flower with petaloid stamens. Interestingly, the expression of mutant features occurs only at low temperature, while at high temperature growth is nearly normal. Characteristics of the *tds* (temperature dependent shooty) mutant are described.

## Materials and Methods

### Origin of the Mutant

Leaf discs from *Nicotiana tabacum* (W38) were used for *Agrobacterium tumefaciens*-mediated transformation. The binary vector pPEV (Lindbo and Dougherty, 1992) containing the yeast *FRE1* gene (Dancis et al., 1992) was introduced into *Agrobacterium* EHA105 (Hood et al., 1993) via a freeze-thaw procedure (Hofgen and Willmitzer, 1988). Standard leaf disc transformation procedures were followed (Horsch et al., 1988) and transformants selected on 400 mg/L kanamycin. Seed from a selfed primary transformant was planted in the greenhouse (22°C day and 19°C night) and both normal and mutant plants were observed.

For *in vitro* germination, seeds were disinfested in 70% ethanol (30 sec.) and 20% Clorox/0.1% Tween 20 (20 min.), rinsed in sterile $dH_2O$,

and placed onto Murashige and Skoog (1962) medium without growth regulators (MS-0) in Magenta boxes (50 ml medium per box). The cultures were kept at 18°C for four weeks. The numbers of normal vs. mutant progeny were determined.

**Growth Analyses Under Controlled Environment**

Selfed seeds from the mutant-producing primary transformant and control wild type (WT) seeds were germinated for 10 d on MS-0 medium in Magenta boxes at 25°C. After 10 d, culture vessels were moved into a 21°C growth chamber (16h light, 10 $\mu$mol.m$^{-2}$.s$^{-1}$) for 16 d to allow identification of *tds* seedlings. To determine effects of temperature on *in vitro* seedling development, mutant and control seedlings were transferred to fresh MS-0 medium (five per Magenta box) and placed into 21°C and 30°C incubation chambers.

Mutant seedlings ($S_1$) were also transferred to soil and placed into a large 30°C Sherer growth chamber (16 h light, 40 $\mu$mol.m$^{-2}$.s$^{-1}$) to allow flowering and seed set. Seeds ($S_2$) from these plants were used for subsequent experiments determining the effect of temperature on the phenotype of the mutant.

WT and mutant $S_2$ seeds were germinated on MS-0 at 25°C. Individual plants were transferred to soil and acclimated for two weeks before introducing them into 30°C and 21°C chambers (16h light, 40

$\mu$mol.m$^{-2}$.s$^{-1}$). Two repeat experiments were performed, with five plants for each treatment. Plant height was measured weekly.

**Anatomy**

For paraffin sections, leaf and shoot apices were fixed in FAA (1.85% formalin, 5% acetic acid, 63% ethanol), brought through a dehydration series into 100% tert-butyl alcohol and embedded in paraplast (Fisher Scientific). Sections (10 $\mu$m) were cut from the paraffin blocks, fixed to glass slides with Haupt's adhesive and dried overnight. Staining was achieved with Johansen's Safranin (in 50% ethanol) and Fast Green (in clove oil).

For plastic sections, leaf tissue was fixed in Karnovsky's solution (Karnovsky, 1965), dehydrated to 100% ethanol and embedded in glycol methacrylate. Sections (3-4 $\mu$m) were fixed to glass slides by heating and stained with Toluidine Blue (0.5% in $dH_2O$).

**Fatty Acid Analysis**

Fatty acid methyl esters were prepared from leaves by heating 50 mg samples for 1h at 80°C in 1 ml 2.5% v/v $H_2SO_4$ in methanol (Browse et al., 1986). An internal standard of 75 ng/$\mu$l lauric acid (Nuchek Co.) was added prior to sample heating. Water (1.5 ml) and hexane (0.5 ml) were added to the vials and fatty acid methyl esters were recovered from the organic phase after vortexing and centrifugation. Samples were derivatized with diazomethane prior to analysis via GC-mass spectroscopy (Finnigan

4000 with Model 4500 source and Varian 3400 GLC). Dilutions of derivatized standards (Nuchek Co.) were used for determination of the plant fatty acid profile.

**Grafting**

Rootstocks ( about 1 cm diameter) were prepared by cutting off the shoot above at least two healthy basal leaves. A vertical incision, 2-3 cm long, was made in the center of the internode and the base of the scion was trimmed to a wedge. Cambia of stock and scion were aligned along the length of the cuts. After securing with paraffin film, a plastic bag was attached to the top of the plant (covering the graft union) and left on for two weeks.

**Effects of Cytokinin and Auxin**

The effects of 6-benzylaminopurine (BAP) and $\alpha$-napthalene acetic acid (NAA) on organogenesis from leaf pieces were determined. Stocks of BAP and NAA were prepared in dimethyl sulfoxide (DMSO) and added to medium after autoclaving (with a final concentration of 0.1% DMSO in the medium)(Schmitz and Skoog, 1970). All combinations between BAP (0, 0.1, 1 and 10 $\mu$M) and NAA (0, 0.1, 1 and 10 $\mu$M) were tested. Leaf discs (1 $cm^2$) from *tds* and WT seedlings (grown on MS-0 medium at 25°C) were placed onto the medium (25 ml in petri dishes) and incubated at 21°C or 30°C.

**T-DNA Analyses**

Genomic DNA was isolated from 2 g of *tds* leaf tissue (Doyle and Doyle, 1990), polysaccharides were removed (Murray and Thompson, 1980) and 10 $\mu$g DNA digested with *Eco*RV. The suggested protocol was followed for denaturation and capillary transfer of digested DNA from a 1% agarose gel to nylon Zeta-Probe membrane (Bio Rad). $\alpha^{32}$P dCTP-containing probe was synthesized using a multiprime kit (Amersham) and a template covering the entire T-DNA region. Autoradiography was performed using Hyperfilm-MP (Amersham). PCR primers specific to the 5′ and 3′ ends of *FRE1* (Dancis et al., 1992) were used to examine the presence of a complete *FRE1* gene in *tds* mutants. Forty cycles of 92°C, 2 min; 52°C, 2 min; 72°C, 3 min were performed using an Ericomp thermocycler.

## Results

**Origin and inheritance**

Selfed ($S_1$) progeny from a *N. tabacum* transformant produced a segregating population of normal and shooty mutant plants (Fig. 3.1 A). When seedlings in Magenta boxes were placed at 21°C for four weeks, 294 normal and 86 mutant phenotypes were scored, a good fit to a 3:1 ratio. Further confirmation of the recessive nature of this trait came from the recovery of uniformly shooty progeny from selfed seed of mutant plants grown at permissive temperatures.

**Fig. 3.1 A-F.** Greenhouse-grown *tds* mutant (**A**), multiple meristems on *tds* shoot apex (**B**), flowers of *tds* (left) and WT (right) with the corolla removed (**C**), *tds* flower showing corolla and petaloid stamens (**D**), plants grown in growth chambers for 49 d, from left to right: WT at 30°C, *tds* at 30°C, WT at 21°C, and *tds* at 21°C (**E**), and *tds* plant grown at 30°C and then 21°C (**F**).

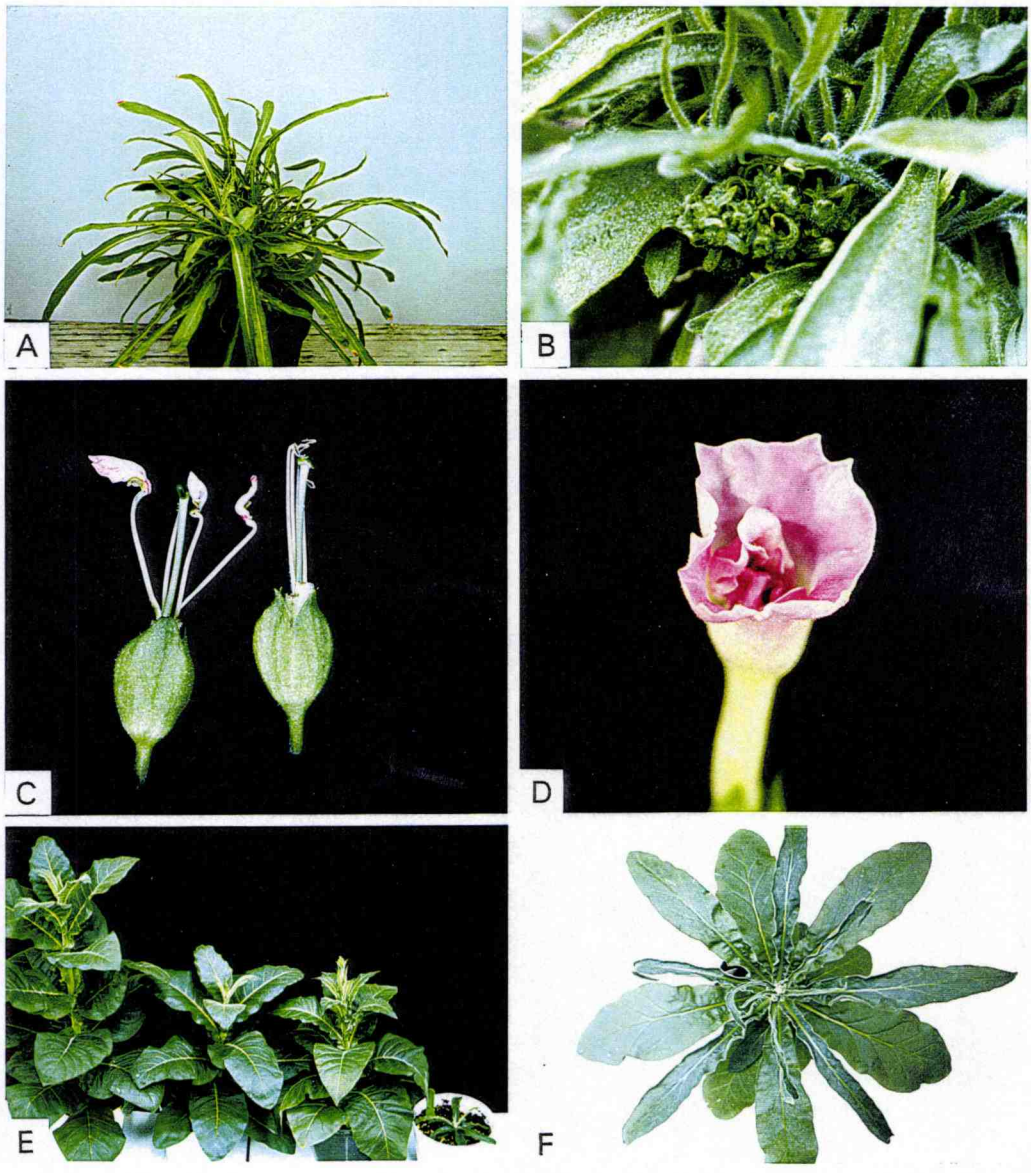



Figure 3.1.

**Morphology**

The most striking characteristics of the mutant are the long and narrow, but thick, almost succulent leaves (Fig. 3.1 A) and the multiple shoots (Fig. 3.1 B). The latter is indicative of a near absence of apical dominance. An occasional adventitious shoot was formed on the leaf surface. Most mutant plants have remained vegetative for more than two years in the greenhouse, while WT tobacco plants usually flower and senesce within six months. The mutant plants rarely flowered, but in such exceptional cases petaloid stamens (Fig. 3.1 C) filled the floral tube, giving the appearance of a double flower (Fig. 3.1 D). Root systems of *tds* mutants were not as well developed as those of normal plants.

Anatomical studies of shoot apices revealed two types of abnormalities in the mutant (Fig. 3.2). The first is the occurrence of multiple meristematic regions (Fig. 3.2 A). Note that the magnification of this apex is the same as that of the WT in Fig. 3.2 B. The other abnormality is necrosis of the apical meristem tip. Newly formed apices appear normal (Fig. 3.2 C), but subsequently they elongate and become more pointed (Fig. 3.2 D), and eventually necrotic (Fig. 3.2 E). At this stage, lateral meristems start growing and the process is repeated.

**Effects of temperature**

The first indication that temperature may influence mutant expression came from the observation that all $S_1$ seedlings grown *in vitro* at 25°C

**Fig. 3.2.** Longitudinal sections of *tds* shoot apex showing multiple meristems; x 24 (**A**), WT shoot apex; x 24 (**B**), and various types of *tds* shoot apices; x 76 (**C-E**). Bar = 100 $\mu$m.

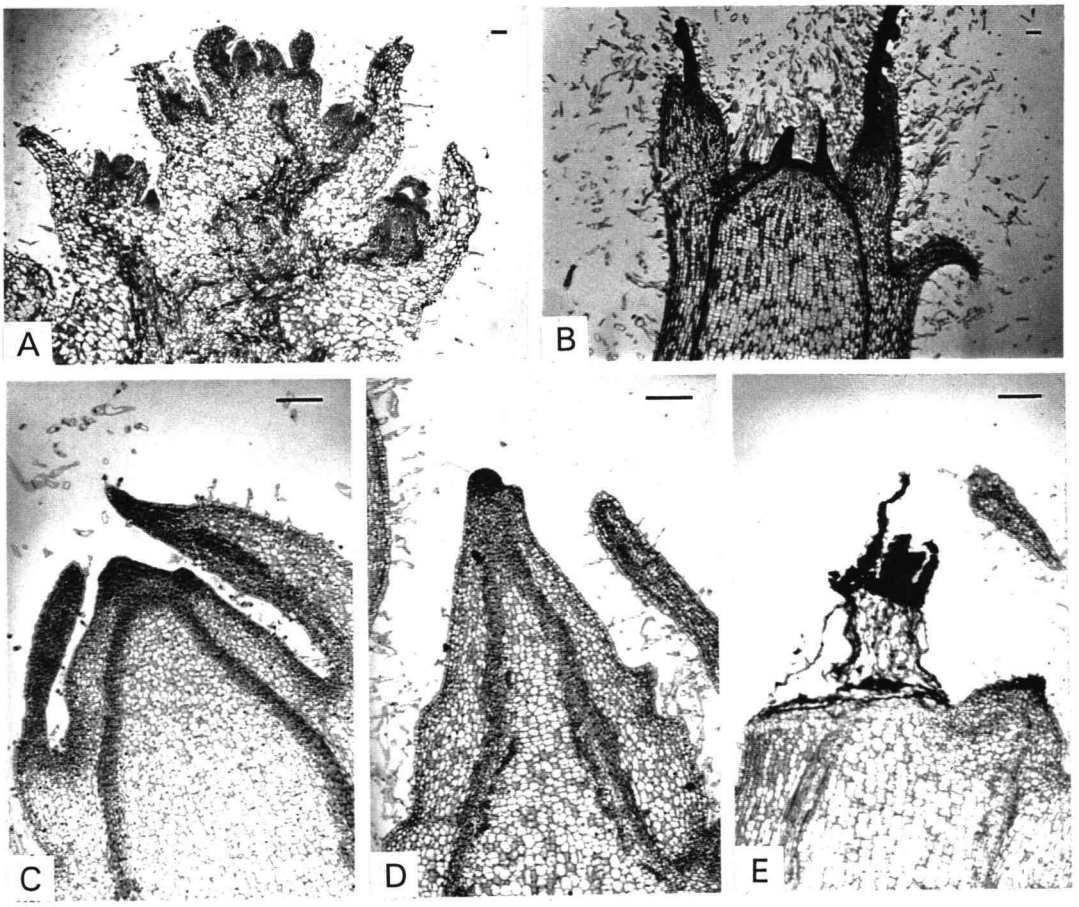



Figure 3.2.

were normal. This led to a series of experiments to examine the effects of temperature in more detail, both *in vitro* and in growth chambers.

The development of mutant seedlings *in vitro* differed dramatically between high and low temperature (Fig. 3.3). At 28°C they developed normally and had broad leaves (Fig. 3.3 A); in contrast, at 21°C they grew slowly and had extremely narrow leaves (Fig. 3.3 B). Also the later development, determined in growth chambers, differed significantly with temperature (Fig. 3.4). At 30°C, the mutants appeared normal, although they were slightly shorter than the WT plants (Fig. 3.1 E and Fig. 3.4). At 21°C, the mutants grew very slowly, had narrow leaves, and exhibited the shooty phenotype. The mutants grew at about the same rate at 30°C as the WT at 21°C (Fig. 3.4). WT plants flowered at low temperature, but the mutant remained vegetative. In contrast, the high temperature inhibited flowering in the WT but induced flowers on the mutant. The only abnormality of mutant flowers was the occurrence of petaloid stamens, similar to the occasional flower formed on mutants in the greenhouse (Figs. 3.1 C and D). Although seed set was low, some viable seed was obtained from the mutants.

The developmental pattern in the mutant seems to be determined by the temperature to which meristems and developing leaves are exposed. Switching mutant plants from high temperature to low temperature resulted in formation of progressively narrower leaves (Fig. 3.1 F). Intermediate

**Fig. 3.3 A, B.** *tds* seedlings in Magenta boxes after 27 d at 28°C (**A**), and 21°C (**B**).

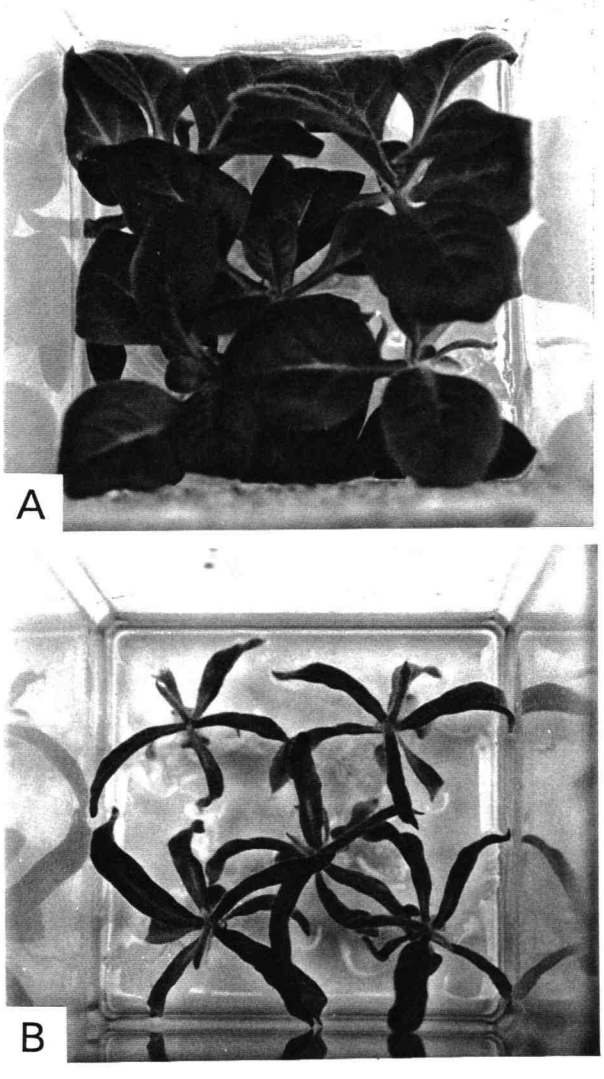



Figure 3.3.

**Fig. 3.4.** Plant height of WT and *tds* grown at 21°C and 30°C. Vertical bars indicate standard deviations from the mean.

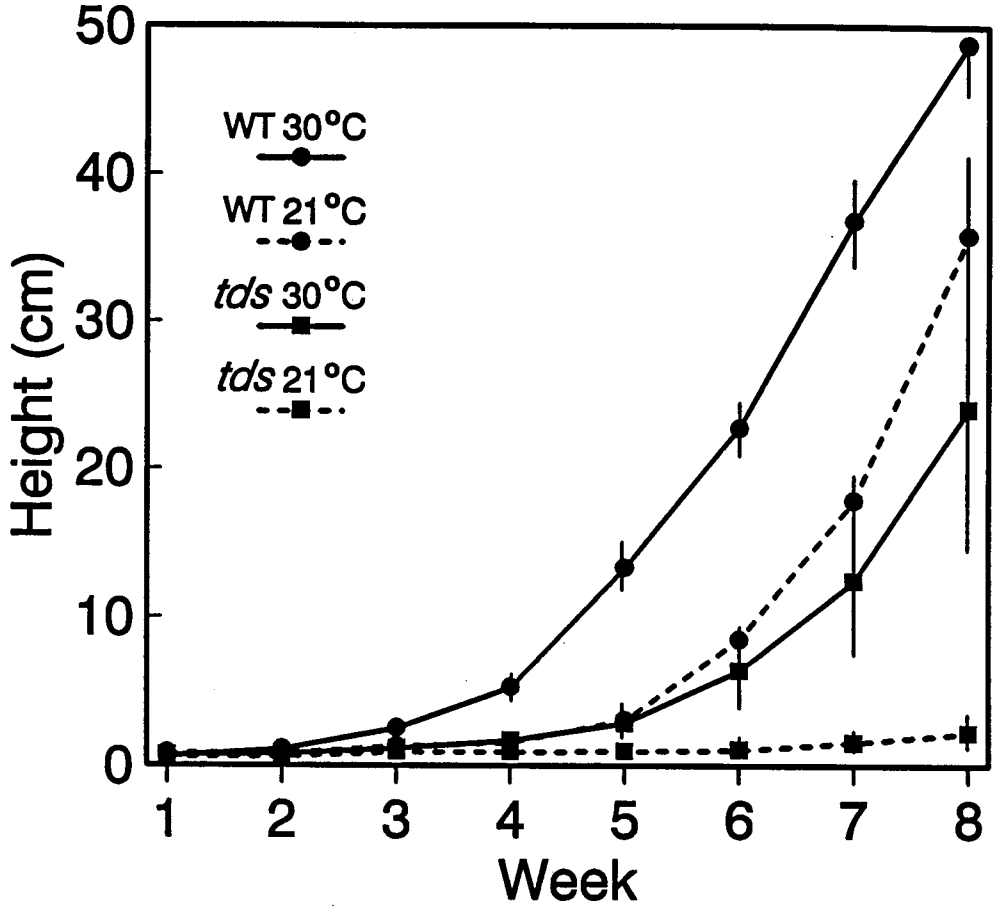

50
40
30
20
10
0
Height (cm)
WT 30°C
WT 21°C
tds 30°C
tds 21°C
1
2
3
4
5
6
7
8
Week

Figure 3.4.

leaves were the result of exposure to high temperature during very early development and low temperature in the later stages.

**Leaf anatomy**

The thick leaves of the mutant, combined with the uneven light transmission (visible when leaves were placed on a light box (Fig. 3.5)), suggested anatomical abnormalities. Leaf sections of mutant and WT tobacco, grown at 21°C and 30°C, were examined. In mutant leaves, tissue resembling the spongy mesophyll occurred where normally the palisade layer is formed (Fig. 3.6). The abnormality was apparent only in plants grown at low temperatures (Figs. 3.6 A and C), but not at high temperatures (Fig. 3.6 E).

**Grafting, growth regulators, and fatty acids**

To determine whether a transmissable factor was responsible for the shooty phenotype, reciprocal grafts between WT and shooty plants were made. None of the combinations effected changes in development. The responses to exogenous cytokinin and auxin, as judged by organogenesis of leaf discs from normalized mutants (grown at 30°C) vs. wild type (also grown at 30°C) was examined at both high and low temperatures. These interaction experiments suggested that there is no major shift in auxin or cytokinin production. However, mutant leaves underwent organogenesis much more slowly at lower temperatures as compared to the WT explants.

**Fig. 3.5.** WT (left) and *tds* (right) leaves photographed on a light box.

Figure 3.5.

**Fig. 3.6 A-F.** Cross sections of leaves from *tds* **(A,C,E)** and WT **(B,D,F)**. Plants grown for 9 weeks at 21°C, paraffin embedded; x 76 **(A,B)**, for 6 months in the greenhouse, plastic embedded, x 190; p = palisade parenchyma **(C,D)**, and for 9 weeks at 30°C, paraffin embedded; x 76 **(E,F)**. Bar = 100 $\mu$m.

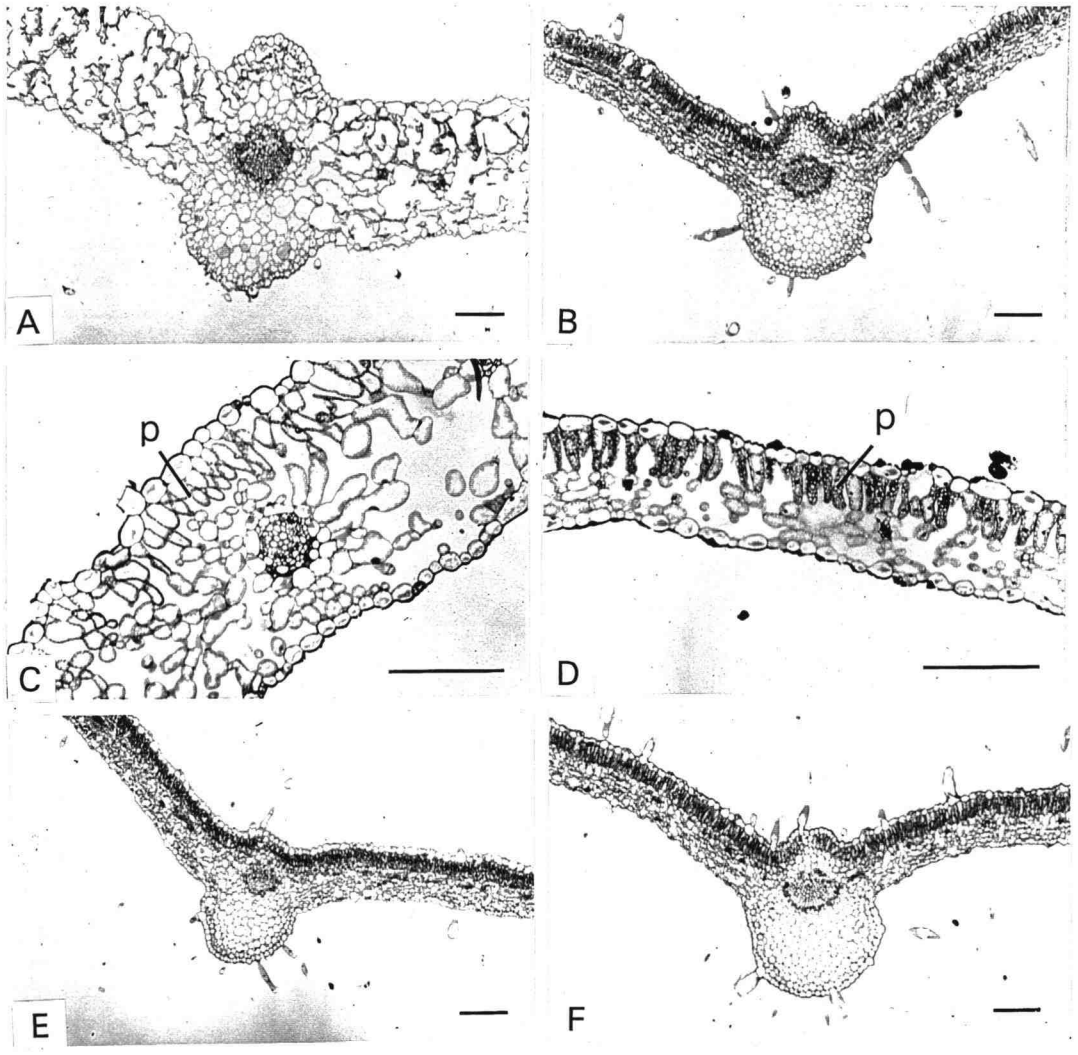



Figure 3.6.

Fatty acid analysis showed no major differences between the mutant and WT profiles (Table 1).

**Search for T-DNA**

PCR and Southern analyses showed the presence of *FRE1* in some mutant plants, but not in others. As expected, mutants with the *FRE1* gene, as detected by PCR, were resistant to kanamycin, whereas those without the gene were susceptible. Thus, the *tds* phenotype seems to be independent of the transgenes. As the probe for Southern blots spanned the entire T-DNA region, the mutation can not be caused by insertion of large T-DNA fragments. However, the presence of a small portion of the T-DNA as a result of partial transfer or excision of T-DNA can not be excluded at this time.

**Table 3.1.** Fatty acid levels of the *tds* and WT plants (μmol/g fresh weight).

| Plant | Fatty acid | | | |
|---|---|---|---|---|
| | Palmitate (16:0) | Stearate (18:0) | Linoleate (18:2) | Linolenate (18:3) |
| *tds* | 5.4 | 0.45 | 5.8 | 14.6 |
| WT | 5.3 | 0.45 | 5.8 | 14.6 |

## Discussion

A recessive morphological mutant has been identified in tobacco. Elevated temperatures normalize the mutant phenotype. It has been suggested that temperature-sensitive lesions arise from point mutations, or other aberrant processing functions, which serve to decrease thermal stability of the gene products (Schnall et al., 1991). The *fab2* mutant of *Arabidopsis thaliana* (Lightner et al., 1994) displayed miniature growth that correlated with an increase in the membrane fatty acid, stearate. High temperature corrected the mutant phenotype, but did not change the fatty acid composition. These observations suggest a possible role for membrane structure in the production of the aberrant morphology. This does not appear to be the case with the *tds* mutant, since the fatty acid profile did not show a difference from that of the WT.

Increased levels of cytokinins can have dramatic effects on plant development. Transgenic tobacco with a constitutively expressed isopentenyl transferase (*ipt*) gene were shooty and did not root (Klee et al., 1987). Medford et al. (1989) placed the *ipt* gene under control of a heat-inducible promoter, causing the release of axillary buds, decreased height, reduced stem and leaf area and an underdeveloped root system in heat-shocked transgenic tobacco. Other approaches using *rolC*, which is suggested to encode a cytokinin-ß-glucosidase, have led to transgenic tobacco that were dwarfed, with thin and lanceolate leaves, had reduced

chlorophyll content, were male sterile, and showed increased root growth *in vitro* (Estruch et al., 1991). The *tds* mutant displays several of these characteristics including narrow leaves, dwarf stature (Fig. 1 A) and an underdeveloped root system. Based on the results of the auxin-cytokinin interaction experiment and grafting, *tds* does not seem to be related to severe hormonal imbalance. However, since the mutant did not show much organogenic response at low temperature, a lesion in hormone perception can not be ruled out.

Medford et al. (1992) described the Schizoid (*Shz*) mutant of *Arabidopsis*, which forms multiple vegetative shoot apices. Sixteen-day-old plants showed necrosis in the main stem cells of the apex and axillary meristems began to grow. It was suggested that this necrosis prevents transmission of an inhibitory signal from the apex, leading to release of axillary buds and the *Shz* phenotype. The *tds* mutant plants also exhibit tip necrosis and growth of lateral shoots. However, suppression of apical dominance in the *tds* mutant is not entirely due to apical tip necrosis since lateral shoots were also formed when apices appeared normal.

Recently, different homeodomain proteins have been overexpressed in tobacco, and interesting pleiotropic alterations in vegetative and/or floral development were observed. Many genes sharing a conserved 183-bp nucleotide sequence known as the homeobox, which encodes the DNA-binding homeodomain, play important roles in developmental controls of cell specification and pattern formation (Scott et al., 1989). Overexpression of

the maize homeobox gene *Knotted-1* (*Kn1*) in tobacco caused a switch from determinate to indeterminate cell fates (Sinha et al., 1993). Phenotypes were variable and depended on the level of KN1 protein, but included dwarfing with rumpled or lobed leaves, lack of apical dominance, ectopic shoot formation on leaf surfaces, and changes in leaf mesophyll. Leaves were roughly twice as thick as the WT and showed either a disorganized palisade parenchyma layer, absence of this layer, or large cells with no distinction between palisade and spongy parenchyma. Leaves of *tds* at low temperature (Fig. 6) resemble KN1 overproducers and produced ectopic shoots in rare cases. Transgenic tobacco plants expressing *Athb-1* (a homeobox gene from *Arabidopsis* of unknown function) showed abnormal development of the palisade parenchyma layer (Aoyama et al., 1995). Sectors of "spongy-like" cells formed in places where normal columnar palisade cells should be found. Likewise, the *tds* mutant also had an abnormal palisade layer (Fig. 6). When *ZmHox1a* and *ZmHox1b*, *Zea mays* homeobox genes, were overexpressed in tobacco, transgenic plants showed a variety of phenotypes including size reduction, leaf narrowing, release of axillary buds and homeotic floral transformations, including formation of petaloid stamens (Uberlacker et al., 1996). However, leaves have not been sectioned to determine if any changes in the palisade layer occurred (W. Werr, personal communication). These plants also have a great deal in common with the *tds* mutants, particularly the floral aberrations and alterations in the vegetative plant body. Also, expression

of the antisense Nicotiana tabacum *AGAMOUS* (*NAG1*) floral organ identity gene in tobacco resulted in petaloid stamens (Kempin et al., 1993). These stamens appear to be virtually identical to those seen in transgenic *ZmHox1* and *tds* flowers. NAG1 contains a MADS box (Schwarz-Sommer et al., 1990) which suggests it acts as a transcription factor (Weigel and Meyerowitz, 1994).

In conclusion, the *tds* mutant shares many features with plants overexpressing homeodomain proteins. However, a critical difference resides in the fact that overexpression of homeodomain proteins behaves as a dominant trait, whereas the *tds* mutant is recessive. Thus far, I have been unable to detect a T-DNA tag in *tds* mutants, but this does not exclude the possibility of incomplete insertion. Aborted T-DNA transfer, resulting in a nick or short T-DNA transfer, can not be ruled out at this time. In any event, the *tds* mutant should be useful for studies of the effect of temperature on the global control of plant development.

# CHAPTER IV

## SUMMARY AND CONCLUSIONS

The uptake and storage of Fe are highly regulated processes and one key mechanism utilized by plants to make Fe available for absorption is the reduction of Fe(III) to Fe(II) via an inducible, PM-bound Fe(III) reductase. Plant genes encoding Fe(III) reductases have not yet been isolated. Yeast shares Fe uptake strategies with plants, in that Fe is first reduced before interacting with a Fe transporter for internalization by the cell. Yeast Fe(III) reductase genes have been cloned. Tobacco was used as a model system to examine the effects of transformation with yeast Fe(III) reductase genes. *Agrobacterium* incorporated *FRE1* and *FRE2* into the plant genome, and Fe(III) reductase activity was measured in homozygous transformants containing *FRE1*, *FRE2*, or both. Homozygous lines containing only *FRE1* or *FRE2* differed in Fe(III) reduction, and some lines had higher Fe(III) reduction than the control. The highest Fe(III) reduction levels were found in lines containing both *FRE1* and *FRE2*, and the results from liquid reductase assays suggested three to four times more Fe(III) reduction in these transformants as compared to controls. Visual plate assays, in which roots were embedded in semi-solid assay reagents, showed reduction along the entire length of the roots. One *FRE1*-containing line initially exhibited chlorosis on medium with low Fe at pH 7.5, but later recovered. Other transformants and the control remained chlorotic on this medium.

To my knowledge, this is the first report that co-expressed FRE1 and FRE2 may increase Fe(III) reductase activity in plants. Crosses between homozygous *FRE1* and *FRE2* lines, with differing numbers of transgenes, are in progress, and the resulting progeny should help determine gene dosage effects. The one line which eventually recovers on high pH, Fe-deficient medium may have a more efficient proton-pumping capability. It appears that this phenotype is independent of the presence of *FRE1*, and will be examined in more detail in the future.

*Agrobacterium*-mediated transformation often produces mutant phenotypes not associated with stable T-DNA integration. A recessive, temperature-dependent, morphological mutant was found among the progeny of tobacco transformed by *Agrobacterium*. Interestingly, the mutant phenotype is expressed only at low temperature (21°C). Mutant characteristics include formation of thick, narrow leaves with abnormal mesophyll cells and near absence of apical dominance. Most plants remain vegetative, and the few flowers that are formed have petaloid stamens. High temperature (30°C) reverses the mutant phenotype, with formation of normal leaves and restoration of apical dominance (many flowers still retain petaloid stamens). The majority of temperature-dependent mutations are restrictive at high temperatures, so *tds* joins an elite group of mutants which normalize at high temperature.

The *tds* mutant shares many features with plants overexpressing homeodomain proteins, although the nature of the lesion is unknown.

Reciprocal grafting and auxin-cytokinin interaction experiments with cultured tissues do not suggest shifts in hormone balance, although a mutation in hormone perception can not be ruled out. The fatty acid profile of *tds* plants appears to be normal. The inability to detect a T-DNA tag may be due to aborted T-DNA transfer or excision of the transgene, alternatively, tissue-culture itself may have created the mutation. Regardless of the cause, the *tds* mutant should be useful for future studies of the effect of temperature on the global control of plant development.

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