# LOCALIZATION OF THE *ARABIDOPSIS* tRNA NUCLEOTIDYLTRANSFERASE IN PLANT CELLS AND CHARACTERIZATION OF A NOVEL *ARABIDOPSIS* PROTEIN (Gim1p) INTERACTING WITH tRNA NUCLEOTIDYLTRANSFERASE.

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## ABSTRACT

**Localization of the *Arabidopsis* tRNA nucleotidyltransferase in plant cells and characterization of a novel *Arabidopsis* protein (Gim1p) interacting with tRNA nucleotidyltransferase**

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The enzyme tRNA nucleotidyltransferase catalyzes the addition of CMP and AMP residues to the 3' end of immature tRNAs. In eukaryotic cells, the nuclear, mitochondrial and chloroplast genomes all encode tRNAs and tRNA nucleotidyltransferase is therefore required in these compartments as well as in the cytosol. In yeast, one gene codes for the tRNA nucleotidyltransferase that functions in the nucleus, mitochondrion and cytosol. As a single gene coding for tRNA nucleotidyltransferase has been identified in *Arabidopsis*, we were interested in determining whether its gene product(s) is(are) targeted to multiple locations. Protoplast transformation experiments using a green fluorescent protein:tRNA nucleotidyltransferase fusion protein suggests that, as in yeast, both mitochondrial and nuclear targeting information are encoded by the *Arabidopsis* tRNA nucleotidyltransferase. In addition, this protein also appears to be targeted to the chloroplast.

The *Arabidopsis* homologue of the yeast Gim1p which has been shown in a yeast two-hybrid assay to interact with the *Arabidopsis* tRNA nucleotidyltransferase (Gu, 2000), complemented a cold and benomyl-sensitive defect in the yeast *GIM1* gene. The two-hybrid interaction between the *Arabidopsis* Gim1p homologue and the *Arabidopsis*

tRNA nucleotidyltransferase was shown to be dependent on the presence of the yeast Gim5p. This observation may suggest that the interaction between Gim1p and tRNA nucleotidyltransferase requires the Genes Involved in Microtubule assembly Complex (GIMC) of which both Gim1p and Gim5p are components.

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## DEDICATION

I would like to dedicate my thesis to my mother who always supported me but never understood the late hours at the lab.

## ABBREVIATIONS

aa -Amino acid

bp -Base pair

Em -emerald

$H_20$ -Water

g -Gram

GFP -green fluorescent protein

His -histidine

Leu -leucine

mGFP -modified green fluorescent protein

min -minute

OD -optical density

PCR -Polymerase chain reaction

PEG -Polyethleneglycol

rpm -Revolutions per minute

SC -Synthetic complete

TBE -Tris/Borate/EDTA

TE -Tris/EDTA

Trp -tryptophan

YPD -Yeast extract/peptone/dextrose

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

# Introduction

Transfer ribonucleic acids (tRNAs) are low molecular weight molecules with a conserved three-dimensional structure (Soll and RajBahandary, 1995). Their primary role is to act as adaptor molecules in protein synthesis by being the direct interface between the amino acid sequence and the information contained in DNA (Soll and RajBahandary, 1995). The biological properties of tRNAs from biosynthesis to function are well defined.

Before a tRNA can function it must go through a number of maturation events mediated by enzymes. In addition, after maturation, tRNAs interact with other proteins (*e.g.*, ribosomal proteins) as they carry out their functions. I am interested primarily in one enzyme, tRNA nucleotidyltransferase, which catalyzes a specific step in tRNA maturation, and any proteins that may interact with tRNA nucleotidyltransferase during its biosynthesis or functioning. I will begin by placing tRNA nucleotidyltransferase in the broader picture of tRNA biosynthesis.

## 1. Transfer RNA Maturation

Transfer RNA synthesis is a multi-step process in which tRNA genes are transcribed, specific bases on the transcripts are modified and leader, trailer and intron sequences (if present) are removed. Many features of tRNA maturation are widely conserved over great evolutionary distances from archaea to eukaryotes to eubacteria (Martin, 1995). Initially, tRNA genes are transcribed as precursors containing additional

residues at both the 5' and 3' ends that must be removed by processing enzymes (Deutscher, 1984). One well characterized processing enzyme, RNase P (Guerrier-Takada *et al.*, 1983, Woese *et al.*, 1990), functions to form the mature 5' end of a tRNA by cleaving pre-tRNAs at specific sites. All cells that synthesize tRNAs are presumed to contain RNase P (Frank and Pace, 1998). Processing enzymes must also modify the 3'ends of tRNAs. In eubacterial cells this is a simple process involving endonucleolytic cleavage by RNase III and RNase E (Ray and Apirion, 1981a,b, Apiron and Miczak, 1993, Deutscher, 1995) followed by exonucleolytic trimming by additional RNases and polynucleotide phosphorylase (Deutscher, 1990). In eukaryotes, 3' processing is more complex using either exonucleases (Garber and Altman, 1979, Engelke *et al.*, 1985) or endonucleases (Schurer *et al.*, 2001). Characterization of 3' processing in yeast demonstrated that while endonucleases are preferred an additional 3' exonucleolytic cleavage pathway exists (Schurer *et al.*, 2001).

Along with the removal of 5' and 3' flanking regions, bases may need to be modified before a tRNA is functional. Several experiments have been performed that implicate these modifications in the various interactions in which tRNAs are involved (reviewed by Bjork, 1995). Modified nucleosides can be found in all phylogenic domains and in identical positions of tRNAs suggesting a conserved function of some tRNA modifications (Bjork, 1986, Bjork *et al.*, 2001). For example, the 5-methyluridine residue at position 54 ($m^5U54$) is a highly conserved feature of eukaryotic and eubacterial tRNAs and the presence of this modification influences the fidelity and rate of protein synthesis as well as the stability of tRNA (Davanloo *et al.*, 1979, Kersten *et al.*, 1981, Johansson and Bystrom, 2002). Base modifications may occur at various stages of

processing (Deutscher, 1995). For instance, the injection of a $tRNA^{Tyr}$ gene into *Xenopus laevis* oocytes demonstrated that all base modifications except those involving the anticodon region occurred before splicing (Melton *et al.*, 1980). Some tRNAs contain introns and undergo additional steps to remove the introns (Deutscher, 1990). After transcription and processing any tRNAs that lack a 3' cytidine-cytidine-adenosine (CCA) at the acceptor stem must have this added by ATP(CTP): tRNA nucleotidyltransferase.

The addition by tRNA nucleotidyltransferase of CMP and AMP residues onto tRNAs that have an incomplete CCA sequence at their 3' terminus is necessary in protein synthesis because this addition enables the correct positioning of peptidyl tRNA at the P-site and aminoacyl tRNA at the A site (Nagaike *et al.*, 2001, Nissen *et al.*, 2000, Samaha *et al.*, 1995). In *E. coli*, yeast and *Sulfolobus shibatae* CCA addition has been shown to be achieved by the recognition of the elbow region of the tRNA formed by the D- and T-loops (Nagaike *et al.*, 2001, Nissen *et al.*, 2000, Samaha *et al.*, 1995). This explains the ability of the enzyme to add the CCA sequence regardless of the amino acid acceptor specificity. The CCA-adding enzyme, tRNA nucleotidyltransferase, shares some regions of sequence similarity between eubacteria, eukarya and archaea (Shi *et al.*, 1998). In *E.coli*, tRNA nucleotidyltransferase has been extensively studied and is one of the best-characterized tRNA maturation enzymes (Williams and Schofield, 1977). It also has been studied in other organisms such as yeast (Chen *et al.*, 1990), *Sulfolobus shibatae* (Shi *et al.*, 1998, Seth *et al.*, 2002), rabbit (Masiakowski and Deutscher, 1980), rat (Mukerji and Deutscher, 1972), wheat (Dullin *et al.*, 1975) and lupin (Cudny *et al.*, 1978, Shanmugam *et al.*, 1996). In *E. coli*, all tRNA genes encode the 3' CCA sequence and thus tRNA nucleotidyltransferase is not coded for by an essential gene in this organism

(Zhu and Deutscher, 1987). Although tRNA nucleotidyltransferase is not essential in *E. coli* it does have a repair function there (Zhu and Deutscher, 1987). In contrast, tRNA nucleotidyltransferase is essential in some other eubacteria, archaea and in all eukaryotic organisms whose genes lack the CCA sequence (Sprinzl *et al.*, 1998) such that it must be added post-transcriptionally by tRNA nucleotidyltransferase.

## 2. Compartmentalized tRNA maturation

While the reactions described for tRNA maturation take place in prokaryotic organisms, the compartmentalization of eukaryotic cells adds an extra level of complexity to this process. Eukaryotic tRNAs are encoded in the nuclear, mitochondrial and chloroplast (when present) genomes (Sprinzl *et al.*, 1998), therefore, tRNA maturation in eukaryotes is a more complicated process requiring the movement of proteins and RNAs from one intracellular compartment to another.

The mechanism by which tRNAs leave the nucleus has been studied extensively (Kruse *et al.*, 2000) and can be related to what is known about the well characterized mechanisms of mRNA maturation (reviewed in Dreyfuss *et al.*, 2002). The tRNA maturation process involves the association of various proteins and protein complexes (Figure 1) and these proteins may not only assist in modification of the tRNA but also in its migration and transport (Kruse *et al.*, 2000). Upon export many of the associated proteins stay bound to the tRNA while others dissociate (Kruse *et al.*, 2000).

The proteins that interact with tRNAs destined for export from the nucleus are synthesized on cytosolic ribosomes and must, therefore, first be transported into the

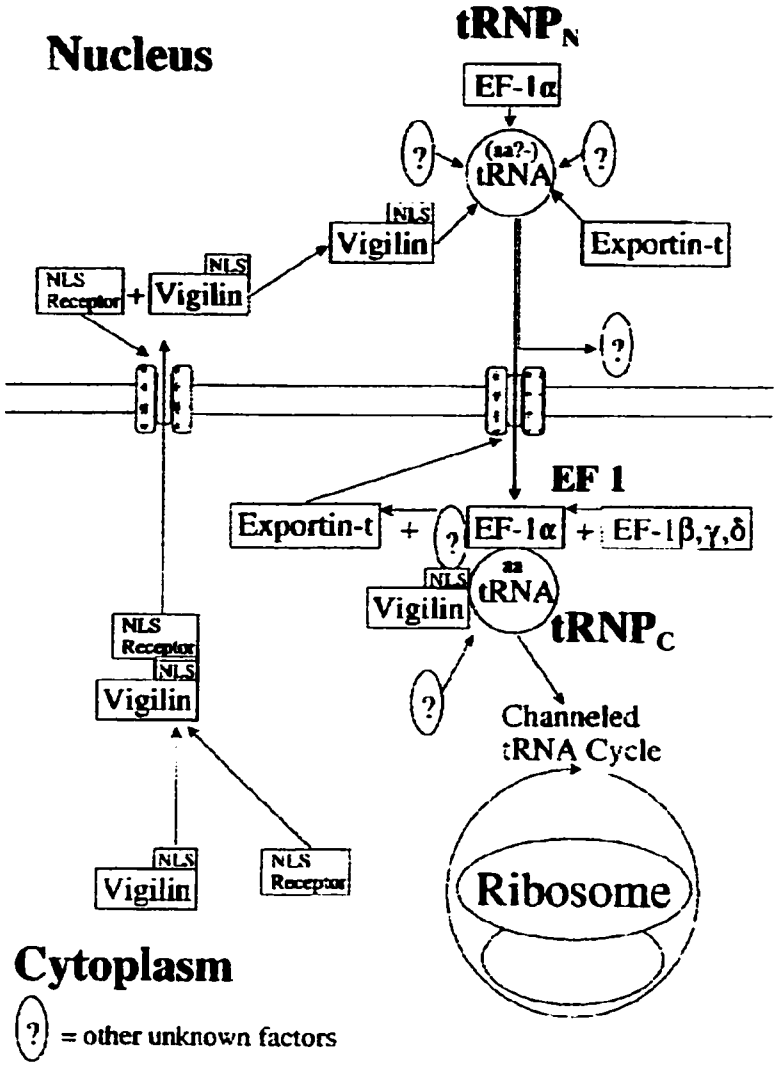


**Figure 1:** A current model highlighting the role of vigilin in tRNA export from the nucleus. Vigilin is presumed to associate with NLS receptors (importin α and β) which mediate import into the nucleus via a nuclear pore complex. Once inside the nucleus, vigilin, associates with other proteins (EF-1 α and exportin-t) and tRNA forming a nuclear transfer ribonucleoprotein complex (tRNP$_N$) for export from the nucleus. Once outside the nucleus some factors dissociate (*e.g.*, exportin-t) and others such as translation factors (EF-1α, EF-1β, EF-1γ and EF-1δ) associate forming the cytosolic tRNP (tRNPc) which can interact with the ribosome to deliver the aminoacyl-tRNA for polypeptide synthesis (Kruse *et al.*, 2000).

nucleus. Vigilin (Figure 1) represents a protein thought to be involved in tRNA export from the nucleus (Kruse *et al.*, 2000). This protein is made in the cytoplasm, translocated into the nucleus, via a nuclear pore complex, by interactions with other associated proteins such as the importin α and β families (Arts *et al.*, 1998, Kutay *et al.*, 1998). Once inside the nucleus it dissociates from this import complex and associates with a tRNA which, in combination with a number of other proteins (*e.g.*, EF-1 α, exportin-t), as well as with other as yet unidentified proteins, can then be exported to the cytosol. Los1p, which is similar to the importin β family of proteins and is the yeast homologue of exportin-t (Sarkar *et al.*, 1998, Feng and Hopper., 2002), has been shown to interact with tRNAs. When the human Los1p homologue, exportin-t, which is 19% similar to the yeast Los1p, was overexpressed in *Xenopus* oocytes and in HeLa cells it facilitated the export of tRNA from the nucleus to the cytosol (Arts *et al.*, 1998, Kutay *et al.*, 1998). As the *LOS1* gene is not an essential gene in yeast (Hurt *et al.*, 1987), there must exist an additional mechanism for tRNA transport. Once tRNA has reached the cytoplasm some proteins (*e.g.*, EF1 and vigilin) remain bound to the tRNA to attract other unidentified proteins while other proteins dissociate from the tRNA complex (Kruse *et al.*, 2000). Clearly, a large number of proteins are involved in tRNA synthesis, maturation and transport. It is possible that some of these proteins will interact with one another in addition to interacting with the tRNA.

Movement of these molecules between the nucleus and the cytoplasm is a signal-mediated process with possible localization signals within the interacting proteins (Feng and Hopper, 2002). In our lab we are interested in the proteins involved in tRNA maturation in general and specifically, in my case, in the properties and targeting of the

CCA-adding enzyme, ATP(CTP):tRNA nucleotidyltransferase, which has been found in the nucleus, mitochondrion and chloroplast.

### 3. Localization and targeting of tRNA nucleotidyltransferase

Although chloroplasts and mitochondria contain their own tRNA genes, many of the proteins involved in the maturation of chloroplast and mitochondrial tRNAs are encoded in the nucleus, synthesized in the cytosol and finally imported into these organelles. One enzyme that is transported into these organelles, as well as into the nucleus, is the modifying enzyme ATP(CTP): tRNA nucleotidyltransferase.

In yeast, one gene (*CCA1*) codes for nuclear, mitochondrial and cytosolic forms of this enzyme (Chen *et al.*, 1992). The *CCA1* gene has three in frame start codons such that initiation of protein synthesis from the different start codons leads to three different forms of Cca1p (Chen *et al.*, 1992, Wolfe *et al.*, 1994). Cca1p-I (protein product from the first start codon) is located primarily in the mitochondrion, but a portion of it is detected in the cytosol and nucleus. In contrast, Cca1p-II (protein product from the second start codon) and Cca1p-III (protein product from the third start codon) are primarily found in the cytosol with a smaller fraction located in the nucleus (Wolfe *et al.*, 1994, 1996).

Recently, the gene (Theologis *et al.*, 2000) and two cDNAs (Gu, 2000, Lam *et al.*, 2001) encoding the *Arabidopsis thaliana* tRNA nucleotidyltransferase have been completely sequenced. The predicted protein products show high similarity with the lupin and yeast forms of the enzyme (Figure 2). Detailed sequence analysis revealed that

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ATCCA1      MILKTMRLSSLPINTLINLPXSLFLISPFRFRNLNRSLTVASRISSTLLR   50
LUPCCA      MRLSFKTVTNVVV--VLPRGRTRSIINFTLFPTITSNLVLHP-----LLR   43
SCERCCA     ML-------------------RSTISLLMNSAAQKTMTN----SNFVLN   26
            *                        *.    ..  .. .       .*.

ATCCA1      VSGVSSRPCGYWFSTNAAMTNVGEEDKQSIPSIELKENIELTDKERKIFD  100
LUPCCA      TPKTPS------F--HSSLSS-------PMSSHKVRDNIQLSDVEKRIFD   78
SCERCCA     APKIT-------------LTKVEQN-----ICNLLNDYTDLYNQK---YH   55
            .. ..              ...          .  ... ..* . .   ..

ATCCA1      RLLSTLRYCNLDTQLRVAGGWVRDKLLGKESDDIDIAIDNMSGSEFLDKF  150
LUPCCA      RLLATLRFFNLQTHLRVAGGWVRDKLLGKECYDIDIALDKMMGTEFVDKV  128
SCERCCA     NKPEPLT-------LRITGGWVRDKLLGQGSHDLDIAINVMSGEQFATGL   98
            .  ..*         **..**********... *.***.. * *..* .

ATCCA1      KEYLSSR----DEEVQGDTVIERNPDQSKHLETAKLRIYDQWIDFVNLRS  196
LUPCCA      REYLLSI----GEEAQGVCVIESNPDQSKHLETARMRLFDMWIDFVNLRS  174
SCERCCA     NEYLQQHYAKYGAKPHNIHKIDKNPEKSKHLETATTKLFGVEVDFVNLRS  148
            .***        ... ..   *..**..*******  ....  .*******

ATCCA1      EEYTENSRIPTM-KFGTAKDDAFRRDLTINSLFYNINSGAVEDLTERGID  245
LUPCCA      EEYTDNSRIPSMQRFGTPEEDAYRRDLTINSLFYNINTDSVEDFTKRGIS  224
SCERCCA     EKYTELSRIPKV-CFGTPEEDALRRDATLNALFYNIHKGBVEDFTKRGLQ  197
            *.**. ****..  ***...** *** *.*.*****....***.*.**.

ATCCA1      DLKSGKIVTPLPAKATFLDDPLRVLRAVRFGARFGFTLDEELKEAASSEE  295
LUPCCA      DLKSGKIVTPLPPKATFLDDPLRVVRAIRFGARFEFTLDEDLKQAAACDE  274
SCERCCA     DLKDGVLRTPLPAKQTFLDDPLRVLRLIRFASRFNFTIDPEVMAEMGDPQ  247
            ***.* . ****.*.*********.* .**..**.**.* ..... .  .

ATCCA1      VRVALGEKISRERIGNEIDLMISGNGPVSAVTYLSDLKLFSVVFALPSSA  345
LUPCCA      VKDALAAKISRERIGTEIDLMISGNQPVKAMTYICDLTIFWIVFSLPPTF  324
SCERCCA     INVAFNSKISRERVGVEMEKILVGPTPLLALQLIQRAHLENVIFFWHNDS  297
            .. *...******.* *.. .. *  *. *.  .    . ..*  . .

ATCCA1      EPSP--PENCGSLSQSYLEAMWSLLKTPRPGKFSGEQRRLA-LYAAMFLP  392
LUPCCA      EPAI--SDGCERLCISQLDISWNLIHLLGKTTFTDEQRRLT-LYAAMFLP  371
SCERCCA     SVVKFNEENCODMD--------KINHVYNDNILNSHLKSFIELYP-MFLE  338
            .      ..*  .          .. .   . .... ...  **. ***

ATCCA1      FRKTVYKDTKGKSIPVVNHIFKFSMKRKTSDAETVMNIHQTTERFRSLIP  442
LUPCCA      LRNTIYREKKAKKVPVVNYIFRESLKRKAKDPETVLDLHRASNKFLSLIP  421
SCERCCA     -KLPILREKIGRSPGFQQNFILSAILSPMANLQIIGNPKKKINNLVSVTE  387
             . .. ... ...    . ..  .. .   . ... . ..  ... *..

ATCCA1      SLEVKKDVELDELTWAADILEHWKSITLNDPVIPATSKIRVLT----GFL  488
LUPCCA      CLVSNEDVQIVGHDWMTELID----------VPVSSRVRVLT----GFL  456
SCERCCA     SI-VKEGLKLSKND-AAVIAKTVDSICSYEEILAKFADRSQLKKSEIGIF  435
            ..  ......   .  . . .          .. .  . *.   *..
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ATCCA1      LRDIKDFWRVSLLTSLLLSATVDGSNDHQDIGQLDFQLERMRETYLTVEA  538
LUPCCA      LRELRDFWRVALLISILLHP-IDVNDTEDESSQLS----KRRDLFNTVEN  501
SCERCCA     LRNFNGHWETAHFAS-LSDAFLKIPKLETKKIELLFQ------NYNEPYS  478
            **.... * .. . * *  . ..  . . .  .*         . .  .

ATCCA1      TIHELGLDKIWDAKPLVNGREIMQIAELKGGSRLIREWQQKLLTWQLAYP  588
LUPCCA      SVIKLGLEKVWDVKQLINGKDVMSVLQLKGGP-MVKEWLDKAMACNLPIP  550
SCERCCA     YIFDNNLNNCHELKPIVDGKQMAXLLQMKPGPWLGKI-NNEAIRWQFDNP  527
             . . .*..  . *....*...  . ..* *. . .   .. . ..  *

ATCCA1      NGTAEECKEWMRDIKAKRQRIE      610
LUPCCA      Q-------ELQRNVL-----IG      560
SCERCCA     TGTDQELITHLKAILPK---YL      546
                   .  ...
```

**Figure 2:** Sequence alignment of the *Arabidopsis* (ATCCA1) lupin (LUPCCA) and *S. cerevisiae* (SCERCCA) tRNA nucleotidyltransferases. The standard one letter code is used for amino acids and the numbers indicate the amino acid position within the protein. Identical amino acids are illustrated with a (*) and similar amino acids by (.). Gaps (-) are introduced to maximize identity. Sequences in bold are not required for enzyme activity and are mitochondrial (SCERCCA) or have characteristics of mitochondrial (ATCCA1, LUPCCA) or chloroplast (ATCCA1, LUPCCA) targeting signals. A canonical nuclear localization signal is underlined in the lupin protein.

like the lupin and yeast CCA-adding enzymes, the *Arabidopsis thaliana* tRNA nucleotidyltransferase could contain a possible amino-terminal organellar targeting signal (Gu, 2000) (bold in Figure 2). Although no nuclear localization signal (NLS) has been identified in the *Arabidopsis thaliana* protein, it seems likely that this protein does contain an NLS in the same relative position as the canonical nuclear localization signal in the lupin sequence (Figure 2). The possibility, therefore exists that, as in yeast, the CCA-adding enzyme in *Arabidopsis thaliana* could be targeted to multiple cellular destinations.

## 4. Protein Targeting

The targeting of proteins to specific cellular compartments has been the subject of intense research and the Nobel Prize in Physiology or Medicine was awarded in this field in 1999. Intracellular transport is essential in the proper function of proteins within the cell. If a protein cannot reach its proper destination, cellular function can be impaired. Targeting signals on a protein as well as additional accessory proteins that recognize these signals allow a protein to go to specific cellular destinations. I will discuss the targeting of proteins with reference to locations in the cell to which tRNA nucleotidyltransferase may be targeted.

### i) Nuclear Targeting

Macromolecules such as proteins and RNAs move in and out of the nucleus through the nuclear pore complex (NPC), which forms a channel for bi-directional

movement (Heese-Peck and Raikhel, 1998). Targeting to the nucleus is a multi-step process. One well-characterized pathway involves binding of the targeted protein to a heterodimeric receptor, followed by translocation through the nuclear pore complex and finally dissociation into the nucleus (Figure 3) (Schlenstedt, 1996, Nigg, 1997). Nuclear-targeted proteins have nuclear localization signals (NLSs) that are characterized by having one or more clusters of basic amino acids which may interact with the importin-β family of proteins along with additional factors for delivery to and through the nuclear pore complex where they can possibly associate with additional factors in export or folding (Dingwall and Laskey, 1991).

**ii) Mitochondria Targeting**

Protein targeting to the mitochondrion is also a multi-step process which involves 1) the synthesis of the precursor protein containing a mitochondrial targeting signal, 2) interaction of the protein with additional factors to induce recognition at the mitochondrial membrane, 3) passing of the protein through the membrane, 4) processing of the precursor protein and 5) assembly of the mature protein (Figure 4) (Zhang and Glaser, 2002). Mitochondrial targeting signals (MTSs) lack acidic residues and contain many basic, hydroxylated and hydrophobic amino acids (von Heijne *et al.*, 1989) and are often found at the amino terminus of proteins. Structurally, MTSs contain two domains: one which can potentially fold into an amphiphilic helix with one positively charged and one apolar face in contact with either lipids or other proteins (von Heijne, 1986) and

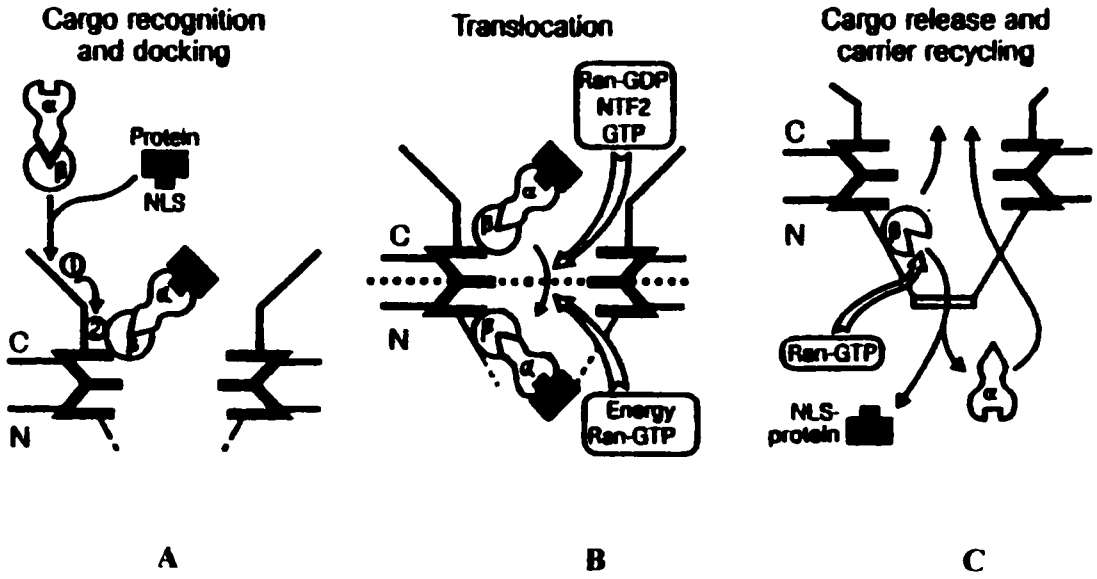



**Figure 3:** Model for the nuclear import of proteins. (A) The recognition of a protein containing a nuclear localization signal (NLS) by an importin α and β heterodimer to form a complex for docking at the nuclear pore complex (NPC). (B) Translocation through the NPC is energy dependant requiring Ran, NTF2 and Ran binding proteins as well as components of the NPC. (C) After translocation both importin α and β dissociate and return to the cytoplasm for additional import. N represents the nucleus and C the cytosol (Nigg, 1997).

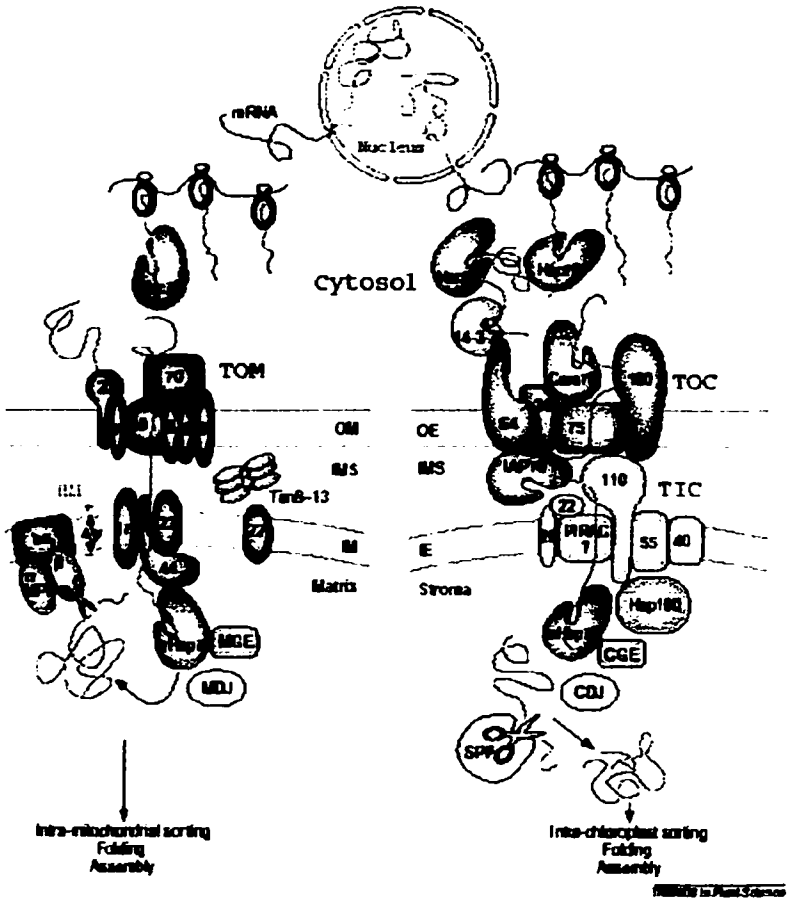



**Figure 4:** Model for protein import into the mitochondrion or chloroplast. Many proteins are synthesized in the cytosol and interact with cytosolic Hsp70 (Hsc70) in cooperation with other chaperones to keep the precursor protein in an import competent state. When proteins are imported into the mitochondrion the Tom complex (Tom 70, 40, 20, 9, 8, 7 and 6) functions as a receptor (Tom 20) and as a channel (Tom 40) for protein import. Once across the outer membrane (OM) the protein interacts with innermembrane space (IMS) proteins (Tim8-13) which assist in its next interaction with innermembrane (IM) proteins (Tim17, 23 and 44) and results in translocation into the mitochondrial matrix. Once in the matrix, mitochondrial Hsp70 (mHsp70) in conjuction with co-chaperones MDJ and MGE (homologues of the bacterial DnaJ and GrpE, respectively) interact with incoming presequences and possibly trigger its unfolding for mitochondrial processing peptidase (MPP). For import of proteins into the chloroplast a similar mechanism occurs with various different proteins. Precursors first are transported to the outer envelope (OE) through the interaction with Hsc70 and 14-3-3 proteins. Once there, they are recognized by Toc 64, transferred first to Toc34 or 160 and finally to the Toc75 channel. The protein traverses through the inner envelope (IE) into the stroma by way of Tic complex (Tic110, 59, 40, 22 and 20). Finally in the stroma it associates with stromal Hsp70 (sHsp70) and possibly CGE and CDJ (bacterial homologues of DnaJ and GrpE respectively) and the targeting signal is cleaved off by stromal processing peptidase (SPP) (Zhang and Glaser 2002).

another which defines a cleavage motif for Mitochondrial Processing Peptidase (MPP). The amphiphilic nature of the MTS indicates that it can interact with the mitochondrial membrane and may be a part of the mechanism of protein import (Glaser *et al.*, 1998). In rat liver mitochondria, the amphiphilic helix is sufficient for import while in soybean mitochondria it is not (Tanudji *et al.*, 1999) suggesting a role for additional proteins in plants. In plants, other steps such as binding to specific chaperones, also may be involved in specificity for mitochondria targeting (Tanudji *et al.*, 1999). Accessory proteins such as Hsp70 and Hsp40 which keep the precursors in an import competent state (Artigues *et al.*, 2002) may be required (*e.g.*, Hsc70 in Figure 4). The mitochondrial membrane contains receptor proteins that recognize precursor proteins in a similar manner to the importin-β family of proteins. These receptors include a Transport Outer Membrane (TOM) complex, which assists in the translocation through the membrane where another set of proteins, Transport Inner Membrane (TIM) complex, complete the transfer through the membrane where finally MPP cleave the protein (Artigues *et al.*, 2002, Neupert and Brunner, 2002, Zhang and Glaser, 2002).

**iii) Chloroplast Targeting**

While import into the chloroplast involves the association of various protein complexes in a similar manner to that of mitochondrial import (Artigues *et al.*, 2002), chloroplast targeting is slightly more complex. It has been shown that import into the chloroplast occurs post-translationally (Schmidt *et al.*, 1986). As with mitochondria the import of proteins into the chloroplast is a multi-step process involving 1) binding of the precursor protein to the organelle surface, 2) translocation between the two envelope

membranes, 3) proteolytic processing of the precursor protein and 4) if necessary, translocation across the thylakoid membrane (Figure 4) (Zhang and Glaser, 2002). Chloroplast targeting signals (CTSs) are generally amino-terminal and lack acidic residues, but contain basic and hydroxylated residues and more serine and threonine residues than do MTSs (von Heijne *et al.*, 1989). Generally, CTSs contain an uncharged amino terminus, a variable central domain containing many serines and a carboxy terminus of 8 to 10 residues, which can potentially fold into an amphiphilic beta-strand (von Heijne *et al.*, 1989). Similar to mitochondrial import, accessory proteins such as Hsc70 (heat shock cytosolic protein 70) and 14-3-3 (a molecular chaperone that modulates interaction between components of the signal transduction pathway) are involved in transporting chloroplast-targeted proteins (Figure 4).

Mitochondrial and chloroplast targeting signals have similar properties but are different enough such that proteins are targeted to their appropriate intracellular destination within the plant and not to the wrong location (Neupert, 1997, Keegstra and Cline, 1999, Filho *et al.*, 1996). However, there are proteins encoded by a single gene that must be targeted to both mitochondria and chloroplasts. For example, glutathione reductase in pea functions in the cytosol, mitochondrion and chloroplast. In this case a single targeting signal directs this protein to both mitochondria and chloroplasts (Creissen *et al.*, 1995). There are other examples of proteins shared between multiple cellular compartments in plants. For example, carrot dihydrofolate reductase-thymidylate synthase, a bifunctional protein involved in dTMP synthesis is found in both the nucleus and chloroplast (Luo *et al.*, 1997). This is done by producing two sets of transcripts from the *DHFR-TS* gene, the long transcript codes for a protein containing a chloroplast

targeting sequence while the short transcript codes for a nuclear version of the protein lacking the chloroplast targeting signal (Luo *et al.*, 1997). Also, *FPS1* (farnesyl-diphosphate synthase) is an *Arabidopsis* gene with two in frame start codons which result in a long transcript generating a protein that is targeted to the mitochondria and the short transcript generating a protein for the cytosol (Cunillera *et al.*, 1997). These two examples show a similar mechanism to what was described previous for targeting of tRNA nucleotidyltransferase to mitochondria and the nucleus in yeast where the additional amino-terminal amino acids code for the targeting information that directs the protein to the mitochondrion. Similarily, in *Arabidopsis*, enzymes involved in tRNA synthesis and function have been shown to be sorting isozymes, *i.e.* a single gene may generate proteins targeted to multiple distinct cellular compartments. For example, valyl-tRNA, threonyl-tRNA, alanyl-tRNA and glycyl-tRNA synthetases are all sorting isozymes and function in the cytosol and mitochondrion while arginyl-tRNA synthetase functions in the mitochondrion, chloroplast and cytosol (see Small *et al.*, 1998 for review). However, as yet no sorting isozyme has been identified that functions in the cytosol, mitochondrion, chloroplast and nucleus. Due to its role in tRNA biosynthesis and repair, tRNA nucleotidyltransferase may represent the first such example.

As described previously, in addition to the targeting information (NLS, MTS or CTS) contained on any specific protein there are a number of accessory proteins that are involved in protein targeting. One class of proteins that has been shown to be involved in this process is molecular chaperones.

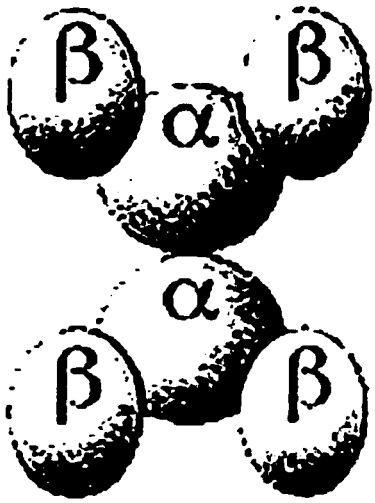
## 5. Molecular Chaperones

The folding process and the correct assembly of many polypeptides are dependent on molecular chaperones (reviewed in Gething and Sambrook, 1992, Hartl, 1996, Bukau and Horwich, 1998). These are proteins that, through their interactions, either prevent the improper folding or assist in the proper folding of various other proteins (Vainberg *et al.*, 1998). Molecular chaperones are of great importance and have many roles in protein folding, protein trafficking (intracellular transport), the interaction of proteins with other cellular components and in conformation changes of some proteins (Hendrick *et al.*, 1993). Chaperonins are one major class of molecular chaperones that have been well characterized. They are involved primarily in assisting protein folding by providing a sequestered environment where folding can proceed unimpaired (Agashe and Hartl, 2000). Chaperonins can be divided into two groups: group I (GroEL-like) found in eubacteria and endosymbiotic organelles (Bukau and Horwich, 1998, Ellis and Hartl, 1999) and group II (TriC/CCT) found in the cytosol of eukaryotic cells (Gutsche *et al.*, 1999 and Willison, 1999). Chaperonins interact with other molecular chaperones to perform various cellular functions by assisting in overcoming kinetic barriers in the folding process (Bukau and Horwich, 1998). Group II chaperonins have been demonstrated to interact with the Prefoldin or GIMC (Genes Involved in Microtubule biogenesis Complex) family of chaperone proteins (Siegers *et al.*, 1999) and also mediate protein folding and are involved in binding to and transferring target proteins to cytosolic chaperonins (Vainberg *et al.*, 1998 and Siegers *et al.*, 1999). Prefoldin or GIMC chaperone proteins have been isolated as heterohexameric complexes from cows

(Vainberg *et al.*, 1998) and yeast (Siegers *et al.*, 1999). The yeast GIMC is assembled as an alpha dimer (Pac10p and Gim5p) surrounded by four beta subunits (Gim1p, Gim3p, Gim4p, and Pfd1p) which may suggest a multi-purpose role for this complex in that each protein in the complex may mediate a different function (Siegert *et al.*, 2000). The Prefoldin or GIMC seems to be conserved evolutionarily because there is a similar complex found in the archaebacterium *Methanobacterium thermoautotrophicum* (Leroux *et al.*, 1999) and eubacteria (Vainberg *et al.*, 1998). The *M. thermoautotrophicum* form of this complex is simplified as compared to the eukaryotic complex because the alpha and beta subunits each contain only one protein (Figure 5) (Leroux *et al.*, 1999).

The GIM complex in yeast has been best characterized through its interaction with TriC (T-complex polypeptide 1 (TCP-1) ring complex) or CCT (Cytosolic chaperonin containing TCP-1), known chaperones that interact in a subunit specific manner with actin and tubulin (Siegers *et al.*, 1999, Geissler *et al.*, 1998). The GIMC protein complex in cooperation with TriC/CCT enables the folding of α- and γ-tubulin, their assembly into a heterodimer and finally the formation of the microtubule (Solomon, 1991). GIMC interaction accelerates actin folding five fold, prevents the early release of non-native proteins from TriC (Siegers *et al.*, 1999) and is essential for cell growth at low temperatures (Geissler *et al.*, 1998). However, there is increasing evidence that these chaperones are not limited to binding actin and tubulin but rather may bind additional unrelated proteins, much like the *M. thermoautotrophicum* GIMC which interacts with not only unfolded actin but also unfolded hen lysozyme, bovine mitochondrial rhodanase and glucose dehydrogenase from *Thermoplasma acidophilum* (Leroux *et al.*, 1999, Siegert *et al.*, 2000) suggesting a more general role for this complex. Since the simplified

# An archael homologue of GIMC/Prefoldin




| Class | Archaea | Eukaryotes |
|---|---|---|
| α | Gim α | Gim2,5 |
| β | Gimβ | Gim1,3,4,6 |

**Figure 5:** Subunit representation of archaeal and eukaryotic homologues of the GIMC (Leroux *et al.*, 1999).

complex in *M. thermoautotrophicum* has multiple functions, this suggests that a more intricate complex, such as in eukaryotic organisms, consisting of different proteins would have even more functions.

Recently, in our laboratory the *Arabidopsis* homologue of one of the subunits (*GIM1*) of this complex was shown through the yeast two-hybrid system to interact with *Arabidopsis* tRNA nucleotidyltransferase (Gu, 2000), therefore, I will focus on *GIM1*.

## 6. GIM1

Gim1p has been best studied in yeast where it is also known as YKE2 (Geissler *et al.*, 1998, Shang *et al.*, 1994). The yeast Gim1p has 114 amino acids and shows significant sequence similarity with mouse KE2 (Figure 6) (Ha *et al.*, 1991, Shang *et al.*, 1994). Geissler *et al.*, (1998) predicted that Gim1p might function in protein folding and assembly, possibly as a molecular chaperone. Yeast cells with the deleted *GIM1* gene have phenotypes, such as supersensitivity to benomyl (a microtubule depolymerizing drug) and cold sensitivity, similar to cells with microtubule defects (Geissler *et al.*, 1998). The supersensitivity results from decreased levels of α-tubulin, which implies that this gene product is involved in tubulin formation and microtubule biogenesis (Geissler *et al.*, 1998). Decreased levels of α tubulin lead to an imbalance in α and β tubulins causing microtubule defects. As microtubules serve to orient the mitotic spindle in yeast (Carminati and Stearns, 1997), this results in the misalignment of the mitotic spindle at cold temperatures, and generates defects in cell division. A yeast *gim1* deletion strain was complemented by the mouse or human *GIM1* homologues (Geissler *et al.*, 1998),

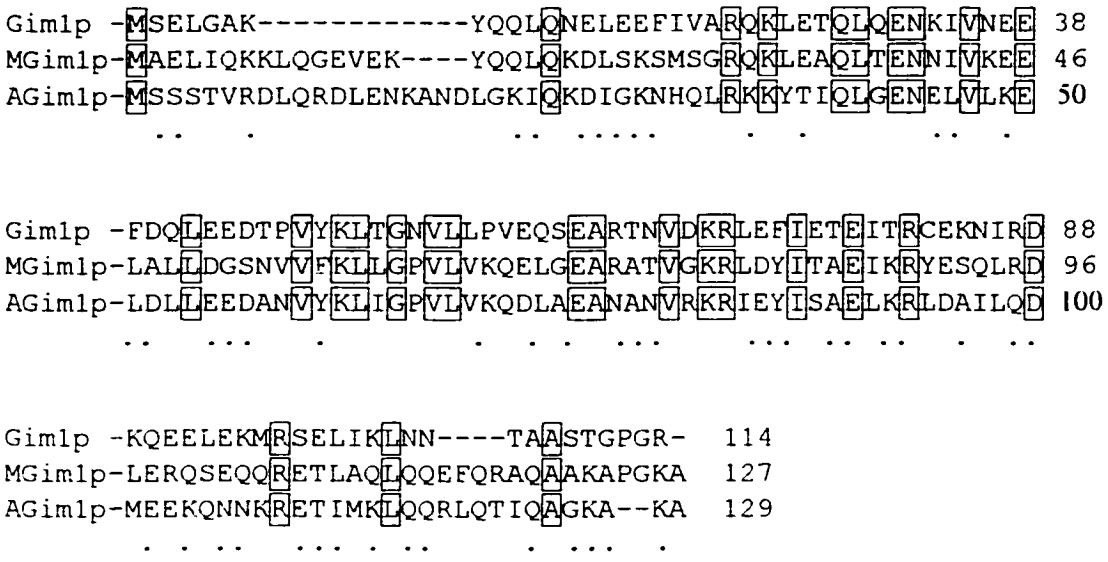



**Figure 6:** Sequence similarity of the yeast, mouse and *Arabidopsis* Gim1 proteins. The predicted *Arabidopsis* Gim1 protein (AGim1p) has been aligned with the mouse GIM1p (Ha *et al.* 1991) and yeast Gim1p (Shang *et al.*, 1994). The standard one letter code is used for the amino acids and the numbers indicate the amino acid position within the protein. Identical amino acids are boxed, similar amino acids are indicated by (.) and gaps are introduced to maximize alignment. In total 29 amino acids are identical (22.1 %) and 53 amino acids are similar (40.5 %).

therefore, suggesting conservation among organisms in the function of this gene product. The GIMC protein complex has been identified in yeast, humans, cows and archaea and it seems likely that a similar complex exists in *Arabidopsis thaliana* since homologues for all of the protein subunits (except pfd1/Gim6p) have been identified at the gene level (Table 1) (Town *et al.*, 2002).

**Table 1: Percent similarity of *Arabidopsis* Gim proteins with homologues from other organisms**

| | Gim proteins | | | | |
|---|---|---|---|---|---|
| Organism | Gim1 | Gim2 | Gim3 | Gim4 | Gim5 |
| *H.sapiens* | 43% / 85 aa | 41% / 182 aa | 39% / 121 aa | 40% / 136 aa | 36% / 135 aa |
| *M.musculus* | 43% / 85 aa | 41% / 182 aa | -[1] | 39% / 140 aa | 36% / 135 aa |
| *A.thaliana* | 100% / 93 aa | 100% / 194 aa | 100% / 128 aa | 100% / 147 aa | 100% / 150 aa |
| *C.elegans* | 43% / 79 aa | 35% / 170 aa | 35% / 116 aa | 27% / 125 aa | 27% / 146 aa |
| *D.melanogaster* | 45% / 92 aa | 37% / 168 aa | -[1] | 35% / 122 aa | 29% / 139 aa |
| *S.cerevisiae*[2] | 50% / 57 aa | 37% / 164 aa | 36% / 123 aa | 31% / 98 aa | 31% / 140 aa |

[1] Indicates homologues have not been identified

[2] *S. cerevisiae* also contains Gim6p which has not yet been identified in any other organism

## 7. This Work

As discussed, in addition to defined targeting signals contained on a protein, many other proteins are involved in directing a protein to its final cellular location. The enzyme tRNA nucleotidyltransferase is needed for tRNA maturation in many compartments in plants. Based on what has been shown in yeast (Chen *et al.*, 1992) and lupin (Shanmugam *et al.*, 1996) it seems likely that the *Arabidopsis* tRNA nucleotidyltransferase is targeted to multiple subcellular destinations. In this work I have attempted to define some of the targeting information contained on this protein and to explore the role of the *Arabidopsis* homologue of the yeast Gim1p which has been shown to interact with tRNA nucleotidyltransferase.

## Materials and Methods

### 1. Strains and growth media

*Saccharomyces cerevisiae* strains SGY101 and HF7c, *Escherichia coli* strains XL2-Blue and JM109 and *Agrobacterium tumefaciens* strain GV3101 were used in this work. The relevant genotypes are listed in Table 2. Yeast strains SGY101 and HF7c were supplied by Dr. Elmar Schiebel and Dr. Pascale Gaudet, respectively. Jason Boyd provided the *Agrobacterium tumefaciens* strain GV3101. *E. coli* strains XL2-Blue and JM109 were purchased from Stratagene and Promega, respectively. Growth media used are listed in Table 3.

### 2. Plasmids

The yeast centromere plasmid p415-ADH (Figure 7), as well as plasmids pSG55 and pSG72 containing the yeast and mouse *GIM1* genes respectively, in p415-ADH, were supplied by Dr. Elmar Schiebel. All of these plasmids contain the *LEU2* gene for selection and the *ADH1* promoter for heterologous gene expression. The *Arabidopsis GIM1* homologue (Figure 6) (with and without a C-terminal 6X His Tag) was cloned into the *Bam*HI and *Hin*dIII sites of the multiple cloning site of p415-ADH to generate pJS19H (with the His tag) and pJS19 (without the His tag).

The plant expression vectors pBIN35SmGFP4-ARACCA-Long (containing the sequence coding for the *Arabidopsis* tRNA nucleotidyltransferase) or pBIN35SmGFP4-

**Table 2: Strains**

| Strain | Organism | Genotype | Reference |
|---|---|---|---|
| JM109 | *E. coli* | *endA1, recA1, gyrA96, thi, hsdR17* ($r_k^-$, $m_k^+$), *relA1, supE44, (lac-proAB), [F', traD36, proAB, laqI$^q$ M15]* | Promega |
| XL2-Blue | *E.coli* | *sup E44 hsd R17 recA1 gyrA46 thi relA1 lac⁻ F' [pro AB⁺ lacI$_q$ lacZ ΔM15::Tn10 (tet$^R$)]* | Stratagene |
| HF7c | *S. cerevisiae* | MATa *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3, 112 gal4-542 gal80-538 LYS2::GAL1-HIS3, URA3::(GAL4 17-mers) CYC-lacZ* | Feilotter *et al.*, 1994 |
| HF7c GIM5::*TRP1* | *S. cerevisiae* | Same as HF7c but with *GIM5::TRP1* | This work |
| SGY101 | *S. cerevisiae* | MAT α *ura3-52 lys2-801 ade2-101 trp1 Δ 63 his3 Δ 200 leu2 Δ 1 Δ gim1* lyke2 :: kanMX4 | Geissler *et al.*, 1998 |
| GV3101 | *A. tumefaciens* | Gentamycin resistance | |

**Table 3: Growth Media and Solutions**

| Organism | Media | Ingredients | References |
|---|---|---|---|
| *E.coli* | YT | 0.8% Bacto-tryptone, 0.5% Yeast Extract, 0.5% NaCl (Plates: 1.5% Agar) | Sambrook *et al*., 1989 |
| *S. cerevisiae* | YPD | 1% Yeast extract, 2% peptone, 2% dextrose (Plates: 2% Agar) | Sherman, 1991 |
| *S. cerevisiae* | Synthetic complete | 20 mg/L adenine, uracil, L-Histidine-HCL, L-Arginine-HCL, L-Methionine<br>30 mg/L L-Leucine, L-Isoleucine, L-Lysine-HCL,<br>50 mg/L Phenylalanine, 200 mg/L Threonine<br>30 mg/L Tyrosine, 20 mg/L Tryptophan<br>0.67% Yeast Nitrogen Base w/o amino acids<br>2% dextrose<br>Plates: 2% Agar | Bai and Elledge, 1996 |
| *Agrobacterium* | Minimal medium<br>20X AB Salt | 5% sucrose<br>20 g/L $NH_4Cl$, 2.9 g/L $MgSO_4$, 3 g/L KCl, 0.2 g/L $CaCl_2$, 50 mg/L $FeSO_4$ $H_2O$ | John Mundy's Lab http://stein.cshl.org/atir/biology/protocols/web_arab/Protocols_Mundy2.ht |
| | 20X AB Buffer | 78.6 g/L $K_2HPO_4$, 23 g/L $NaH_2PO_4$ $H_2O$ | |

**Table 3: Growth Media and Solutions**

| Organism | Media | Ingredients | References |
|---|---|---|---|
| *Agrobacterium* | MS selection media | 4.5 g/L Murashige and Skoog basal salt mixture (Sigma), 50 ml of 20X AB salt and 50 ml 20X AB buffer (plates: 1.5% Agar, Noble grade (Sigma) | http://stein.cshl.org/atir/biology/protocols/web_arab/Protocols_Mundy2.html |
| | Macroelements (10X) | 8.23 g/l $NH_4NO_3$, 9.5 g/l $KNO_3$, 0.85 g/l $KH_2PO_4$, 22.2 g/l $CaCl_2 \cdot 2H_2O$, 1.85 g/l $MgSO_4 \cdot 7H_2O$ | |
| | Iron solution (100X) | 0.74 g $Na_2EDTA \cdot 2H_2O$, 0.556 g $FeSO_4 \cdot 7H_2O$ in 200 mL $H_2O$ | |
| | Microelements (1000X) | 100 mg $MnSO_4 \cdot 4H_2O$, 1 g $ZnSO_4 \cdot 7H_2O$, 1 g $H_3BO_3$, 30 mg $CuSO_4 \cdot 5H_2O$, 10 mg KI, 30 mg $NiCl_2 \cdot 6H_2O$ and 30 mg $AlCl_3 \cdot 6H_20$ in 1 L | |
| | Plasmolysis solution pH 5.5 | 100 ml/l macroelements, 1 ml/l microelements, 10 ml/l of iron solution, 100 ml/l BAP [100 mg/l], 77.4 g/l of mannitol | |

**Table 3: Growth Media and Solutions**

| Organism | Media/Solution | Ingredients | References |
|---|---|---|---|
| | Enzyme solution pH 5.5 | 20 ml macroelements, 200 ul microelements, 2 ml iron solution, 20 ml BAP [100 mg/l], 18.22 g mannitol in 150 ml $H_2O$. Add 3 g cellulysin (Calbiochem), 0.6 g pectinase (Calbiochem) then adjust to 200 ml with $H_2O$ | |
| | Dense solution pH 5.5 | 100 ml/l macroelements, 1 ml/l microelements, 10 ml/l of iron solution, 171.2 g/l saccharose | |
| | Culture medium pH 5.5 | 100 ml macroelements, 1 ml microelements, 10 ml iron solution, 1 ml biotine [10 mg/l], 10 ml nicotine [100 mg/l], 10 ml pyridoxine-HCl [100 mg/l], 10 ml thiamine-HCl [100 mg/l], 10 ml calcium pantothenate [100 mg/l], 10 ml L-cysteine-HCl [100 mg/l], 10 ml inositol [10g/l], 1 ml 2,4-D [100 mg/l], 10 ml NAA [100 mg/l], 2 ml BAP [100 mg/l] | |
| | Electroporation solution (2X) | 0.2383 g HEPES, 0.8766 g NaCl, 0.375 g $CaCl_2$, 6.1030 g mannitol in 100 ml $H_2O$ | |

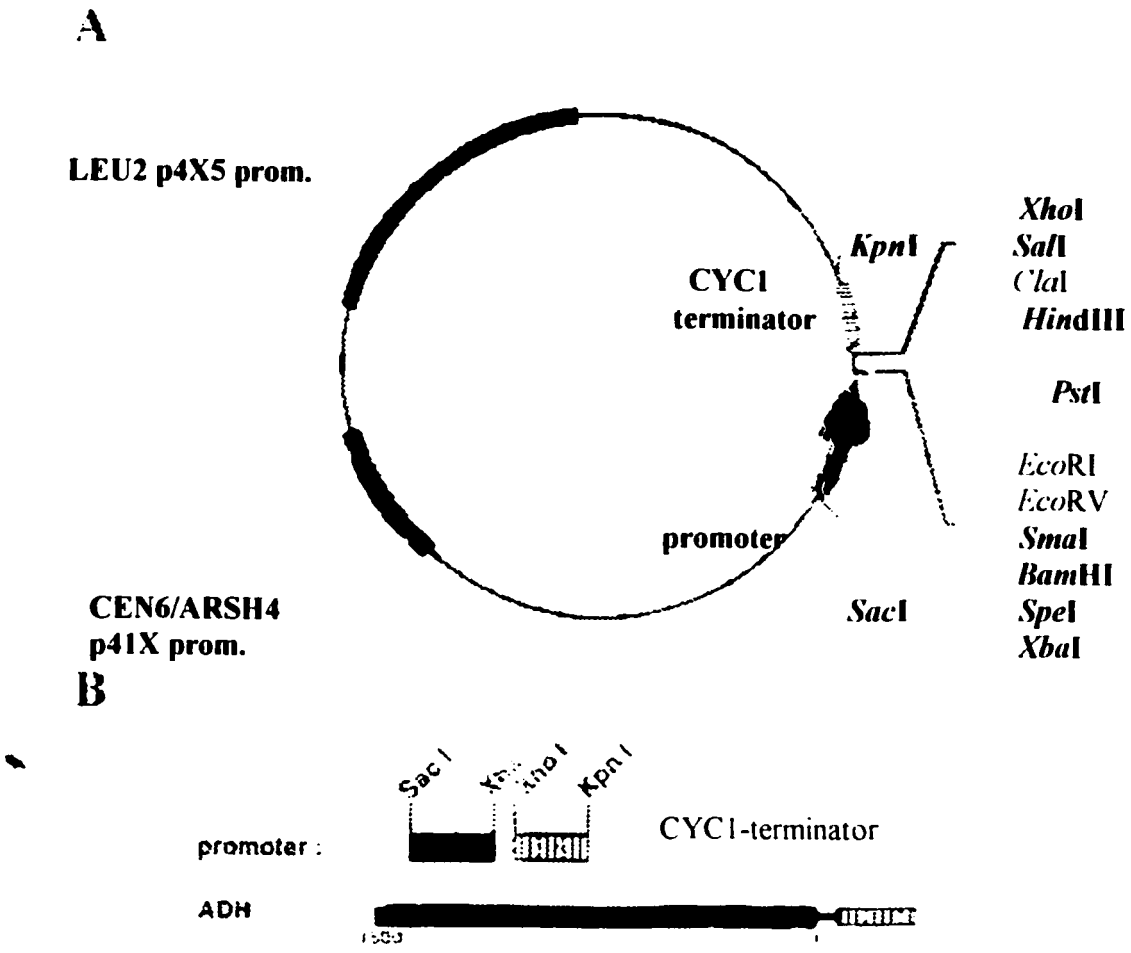



**Figure 7:** Map of p415-ADH expression vector. (A) The plasmid p415-ADH carries the *ADH* promoter and is based on pRS414 carrying the *LEU2* gene and Cen6/ARSH4. The restriction sites of the multiple cloning site are shown between the promoter arrow and terminator and unique sites are in bold. (B) Map of the promoter (dark box) and *CYC1* (striped box) terminator expression cassette (Mumberg *et al.*, 1995).

ARACCA-Short (lacking the targeting signal) fused to GFP (generated by Dr. P. Joyce) and pBIN35SmGFP4-CCA-TS containing only the potential targeting signal (amino acids 1-68 of the tRNA nucleotidyltransferase) fused to GFP were generated by cloning the appropriate inserts into the *Bam*HI site of pBIN35SmGFP4 (Haseloff *et al.*, 1997).

The plant expression vector pBIN35S35S-EmGFP was generously donated by Alexandre Joyeaux (Universite de Montreal). The inserts of interest (CCA-Long, CCA-Short and CCA-TS) were cloned into the *Bam*HI site of this vector generating pBIN35S35SEmGFP-ARACCA-Long, pBIN35S35SEmGFP-ARACCA-Short and pBIN35S35SEmGFP-ARACCA-TS. All plasmids used in this work are listed in Table 4.

**3. Polymerase Chain Reaction (PCR)**

The templates and primers used to generate the PCR products are listed in Table 5. To generate the inserts to construct pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag), pBIN35SmGFP4-ARACCA-TS, pBIN35S35SEmGFP-ARACCA-Long, pBIN35S35SEmGFP-ARACCA-Short and pBIN35S35SEmGFP-ARACCA-TS each PCR reaction contained 1X Native *Pfu* buffer (Stratagene), 200 μM dNTPs, 100 pmoles of each primer, ~10 ng template DNA and 1.25 units of *Pfu* polymerase (Stratagene) in a final volume of 50 μl. The samples were overlaid with two drops of mineral oil and the reaction was performed using a Perkin Elmer DNA thermal cycler. The following reaction conditions were used: 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 45 sec, annealing at 37 to 42°C (depending on the

**Table 4: Plasmids Used in this Work**

| Plasmid | Insert | Source |
|---|---|---|
| p415-ADH | - | Mumberg *et al*., 1995 |
| pSG55 | Yeast *GIM1* | Geissler *et al*., 1998 |
| pSG72 | Mouse *GIM1* cDNA | Geissler *et al*., 1998 |
| pAS2-ARACCA-Long | *Arabidopsis* tRNA nucleotidyltransferase | Gu, 1998 |
| pACT14A | *Arabidopsis* 14Ap cDNA | Gu, 1998 |
| pACT19A | *Arabidopsis GIM1* cDNA | Gu, 1998 |
| pACT108A | *Lupin* 108Ap cDNA | Gu, 1998 |
| pTric-His19A | *Arabidopsis GIM1* cDNA | Gu, 1998 |
| pRS314 | - | Sikorski and Hieter, 1989 |
| pJS19 | *Arabidopsis GIM1* cDNA | This work |
| pJS19H | *Arabidopsis GIM1* cDNA containing sequence coding for 6X His tag | This work |
| pBIN35SmGFP4-ARACCA-Long | *Arabidopsis* CCA cDNA starting from ATG1 | Paul Joyce |

**Table 4: Plasmids Used in this Work**

| Plasmid | Insert | Source |
|---|---|---|
| pBIN35SmGFP4-ARACCA-Short | *Arabidopsis* CCA cDNA starting from ATG3 | Paul Joyce |
| pBIN35SmGFP4-ARACCA-TS | *Arabidopsis* CCA mitochondrial targeting signal (ATG1 to ATG3) | This work |
| pBIN35S35SEmGFP-ARACCA-Long | *Arabidopsis* CCA cDNA starting from ATG1 | This work |
| pBIN35S35SEmGFP-ARACCA-Short | *Arabidopsis* CCA cDNA starting from ATG3 | This work |
| pBIN35S35SEmGFP-ARACCA-TS | *Arabidopsis* CCA mitochondrial targeting signal (ATG1 to ATG3) | This work |

**Table 5: Primer Sequences**

| Oligos | Sequence | Purpose | Restriction site |
|---|---|---|---|
| 19A(F) | 5'ACGACGGGATCCATGAG TTCATCGACTG 3' | 5' end primer of *Arabidopsis GIM1* | *Bam* HI |
| 19A(R) | 5'GAGTAAAGCTTTCAAGC CTTTGCTTTTCC 3' | 3' end primer of *Arabidopsis GIM1* | *Hin* dIII |
| 19A(R )-HIS | 5'TCTAGAAAGCTTTCAGT GGTGATGATGGTGGTGAG CCTTTGCTTTTCCTGCCTG 3' | 3' end primer of *Arabidopsis GIM1* containing a 6X His tag | *Hin* dIII |
| ATG1GFP | 5'GTCGACTGATCATCTAG AATTCAACAATGATACTA AAAACCATG 3' | 5' end primer of *Arabidopsis* CCA from ATG1 | *Bcl* I |
| ATG3GFP | 5'GTCGACTGATCATCTAG AATTCAACAATGACGAAT GTTGGAGAGG 3' | 5' end primer of *Arabidopsis* CCA from ATG3 | *Bcl* I |
| CCA3EMGFP | 5'CTGCAGCTGATCAGCCT CTATCCTTTGTCGTTTAGC 3' | 3' end primer for *Arabidopsis* CCA | *Bcl* I |
| KE2RTF | 5'AGGCTAATGATCTCGGC AAA 3' | 5' end primer of *Arabidopsis GIM1* RT PCR | - |
| KE2RTR | 5ÁGAATTGCATCAAGCCG TTT 3' | 3' end primer for *Arabidopsis GIM1* RT PCR | |
| Y2H-GIM5 (F) | 5'TGCTGTGACCCAAACAA GAAAGAGCACTGTAAAAA TCAAGCCATGAGATTGTA CTGAGAGTGCAC 3' | 5' end primer for *GIM5* disruption with *TRP1* | - |

**Table 5: Primer Sequences**

| Oligos | Sequence | Purpose | Restriction site |
|---|---|---|---|
| Y2H-GIM5 (R) | 5'CAAATGAGATATGAAAA ATCGTAGGAAAAGCAAAA GAGTTACCTACTGTGCGG TATTTCACACCG 3' | 3' end primer for *GIM5* disruption with *TRP1* | - |
| GIM5CONF | 5'TGCTGTGACCCAAACAA G 3' | 5' end primer for confirmation of *GIM5* disruption in yeast | - |
| GIM5CONR | 5'TGAAAAATCGTAGGAAA AGC 3' | 3' end primer for confirmation of *GIM5* disruption in yeast | - |

primers) for 45 sec and elongation at 72°C for 1 to 2 min depending on the size of the expected product. The last cycle was finished with 72°C for 10 min. The specific reaction conditions for each construct are listed in Table 6. PCR amplification of cDNA generated from various tissues was also carried out as described above except using 1 μg of each template and a 55°C annealing temperature.

Yeast colony PCR (Akada *et al.*, 2000) was used to identify gene disruptions in HF7c. Colonies were patched on YPD plates and incubated for 48 h. Approximately 3 $mm^2$ of cells were collected (giving roughly 1 X $10^7$cells), dissolved in 20 μl of 0.25% SDS (sodium dodecyl sulfate), vortexed and centrifuged for 30 sec at 14 000 rpm. For PCR 1 μl of this preparation served as template in 1X Taq buffer (MBI), 1.5 mM $MgCl_2$ (MBI), 0.2 mM dNTPs, 10 pmols of each primer, 1.25 μl 20% Triton X-100 and 1.25 units of Taq polymerase (MBI) in a final volume of 25 μl. The denaturation, elongation and annealing conditions were as described previously.

To generate products for homologous recombination into the *GIM5* gene of the yeast strain HF7c each PCR reaction contained 1 μl of pRS314 template, 1X Taq buffer, 2.5 mM $MgCl_2$, 0.8 mM dNTP, 25 pmols of each primer (Table 4) and 2 units of Taq polymerase in a final volume of 50 μl. The samples were overlaid with 2 drops of mineral oil and the reaction was performed using a Perkin Elmer DNA thermal cycler. The following reaction conditions were used (Brachmann *et al.*, 1998): 94°C for 5 min, followed by 10 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min. This was followed by 20 cycles of denaturing at 94°C for 1 min, annealing 65°C for 1 min and elongation at 72°C for 2 min. The last cycle was finished with 72°C for 10 min.

**Table 6: Polymerase chain reaction conditions**

| Product | Expected | Primers Forward | Reverse | PCR Templates | |
|---|---|---|---|---|---|
| *Arabidopsis GIM1* | ~390 bp | 19A(F) | 19A(R ) | 19A2 cut B+E | Annealing: 45 sec 42°C Extension: 1min 72°C |
| *Arabidopsis GIM1* (with 6X His tag) | ~410 bp | 19A(F) | 19A(R )-HIS | 19A2 cut B+E | Annealing: 45 sec 42°C Extension: 1min 72°C |
| *Arabidopsis* CCA mitochondrial targeting signal (ATG1 to ATG3) | 207 bp | ATG1GFP | CCA3GFP | pAS2-ARACCA-Long | Annealing: 1min 37°C Extension: 1min 72°C |
| *Arabidopsis* CCA cDNA starting from ATG1 | 1895 bp | ATG1GFP | CCA3EM-GFP | pAS2-ARACCA-Long | Annealing: 1min 37°C Extension: 2min 72°C |
| *Arabidopsis* CCA cDNA starting from ATG3 | ~1690 bp | ATG3GFP | CCA3EM-GFP | pAS2-ARACCA-Long | Annealing: 1min 37°C Extension: 2min 72°C |
| *Arabidopsis* CCA mitochondrial targeting signal (ATG1 to ATG3) | 207 bp | ATG1GFP | CCA3EM-GFP-TS | pAS2-ARACCA-Long | Annealing: 1min 37°C Extension: 1min 72°C |

**Table 6: Polymerase chain reaction conditions**

| Product | Expected | Primers Forward | Reverse | PCR Templates | |
|---|---|---|---|---|---|
| *TRP1* gene | ~ 775 bp | Y2H-GIM5 (F) | Y2H-GIM5(R ) | pRS314 | Annealing: 1min 55°C<br>Extension: 2min 72°C<br>Annealing: 1min 65°C<br>Extension: 2min 72°C |
| region of *GIM1* open reading frame | ~ 700 bp | 19A(F) | 19A(R ) | | Annealing: 1min 55°C<br>Extension: 1min 72°C |
| disrupted *GIM5* gene | ~ 1.2 kb | GIM5CONF | GIM5CONR | | Annealing: 1min 55°C<br>Extension: 2min 72°C |
| wild type *GIM5* gene | ~ 700 bp | GIM5CONF | GIM5CONR | | Annealing: 1min 55°C<br>Extension: 2min 72°C |

After completion of each PCR amplification an aliquot (5 µl) of each PCR mix or, in the case of colony PCR, the entire mix was used to confirm by agarose gel electrophoresis the presence of the desired fragment. To the remainder of the sample an equal volume of water and either 10 µl of 3 M sodium acetate or 50 µl of 7.5 M ammonium acetate was added. The sample was mixed, phenol extracted twice with equal volumes of phenol and subjected to ether extraction to remove residual phenol. The sample was air dried for 2 minutes and then precipitated by adding 2 volumes of 99% ethanol with incubation at -70°C for 1 hour. Samples were centrifuged at 14 000 rpm for 30 min at 4°C, decanted, washed with 80% ethanol, desiccated and resuspended in the desired volume (10-50 µl) of sterile water. For gene disruptions, the samples were ethanol precipitated a second time to remove any residual phenol.

**4. Restriction digestions**

The restriction enzymes used were purchased from MBI or NEB. DNA samples (0.2-2 µg) were incubated with 1X reaction buffer and 2-5 units of enzyme in a final volume of 10 µl. The mixture was incubated at the temperatures indicated by the supplier. To generate pJS19 (*Arabidopsis GIM1*) and pJS19H (*Arabidopsis GIM1* with 6X His tag), the PCR products and plasmid (p415-ADH) were digested with *Bam*HI and *Hin*dIII. To construct pBIN35SmGFP4-ARACCA-TS, pBIN35S35SEmGFP-ARACCA-Long, pBIN35S35SEmGFP-ARACCA-Short and pBIN35S35SEmGFP-ARACCA-TS the PCR products were digested with *Bcl* I and pBIN35S35SEmGFP was digested with *Bam*HI.

The plasmids were dephosphorylated to prevent self-ligation. All restriction digestions were confirmed by electrophoresis at 95 volts for 30-60 min on a 1% agarose TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) gel containing 1 μg/ml ethidium bromide.

**5. Phenol freeze fracture** (Bewsey *et al.*, 1991)

In summary, after electrophoresis the DNA fragment of interest was excised from the agarose gel and placed in a 1.5 ml Eppendorf tube to which 300-400 μl of phenol was added. The fragment was vortexed for about 2 min, stored at -70°C for a minimum of 30 min and then thawed at 37°C for 10 min. An additional 300-400 μl of phenol was added, the sample vortexed, placed at -70°C as described previously, and finally thawed again. At this point, 100-200 μl of water and 50 μl of 3 M sodium acetate were added, the sample was vortexed and centrifuged at 14 000 rpm for 10 min at 4°C. The aqueous phase was collected and extracted twice with phenol and twice with ether followed by ethanol precipitation as described previously. Finally, the fragment was resuspended in the desired volume of sterile water (10-20 μl).

**6. Ligation**

Ligations were performed according to the protocol supplied with T4 DNA ligase (MBI). Insert (100-500 ng) and plasmid (10-60 ng) were mixed with 1X ligase buffer, 2.5 units of ligase (MBI) and 0.25 mM ATP in a final volume of 20 μl and incubated at 4°C overnight.

**7. *E. coli* transformation** (Sambrook *et al.*, 1989)

Ligation mixtures (5 µl) or 50 ng of plasmid DNA were incubated for 30 min on ice with 45 µl of competent XL2-Blue cells (prepared by Dr. Pamela Hanic). The cells were then heat shocked at 42°C for 1.5 minutes, an additional 900 µl of YT (Table 3) was added and the cells incubated at 37°C with shaking for 45 minutes. The cells were centrifuged at 14 000 rpm for 1 min, 800 µl of the aqueous phase was removed and the pellet was resuspended in the remaining liquid. The transformed cells were plated on YT agar plates (Table 3) containing the appropriate antibiotic (50 µg/ml Ampicillin or 50 µg/ml Kanamycin) and incubated at 37°C overnight.

**8. Plasmid preparation**

**i) High copy number plasmids**

A single colony was inoculated into a 50 ml Falcon screw cap tube containing 5 ml of YT with the desired antibiotic and incubated overnight at 37°C with shaking at 225 rpm. Plasmid DNA was isolated using the protocol supplied with the commercial SpinPrep Plasmid Kit (Novagen) with the following modification: plasmid was eluted with 2 aliquots of 30 µl of prewarmed (60°C) Elution buffer C at step 12 giving 60 µl of plasmid DNA at a concentration of 1-2 µg/µl.

**ii) Low copy number plasmids** (Krieg and Melton, 1991)

A single colony from the transformation was inoculated into 200 ml YT containing the appropriate antibiotic and incubated overnight at 37°C with shaking at 225 rpm. Plasmid DNA was isolated from the cells using the double stranded DNA template preparation procedure in the Promega protocols and applications guide (1991). Pellets were resuspended in 30-60 µl of sterile $H_2O$ to give a final concentration between 1-2 µg/µl.

**iii) EndoFree plasmids**

Plasmids for transformation into tobacco protoplasts were prepared using the EndoFree Maxi Kit from Qiagen. The procedure was as described in the protocol supplied and the pellet was resuspended in 300 µl of supplied TE (10 mM Tris, 1 mM EDTA).

**9. Preparation of competent yeast cells**

Yeast competent cells were prepared according to Schiestl and Gietz (1989). All steps were performed using sterile technique. A single colony was inoculated into 5 ml of YPD (Table 3) and incubated at 30°C with shaking at 225 rpm until stationary phase (48 h). An aliquot of this (350 µl) was used to inoculate 300 ml of YPD and incubated at 30°C until an $OD_{640}$ of 0.2-0.9 was reached. The culture was centrifuged at 5000 rpm for 10 min at room temperature using a JA-14 rotor in a Beckmen J2HS centrifuge. The pellet was washed twice with 150 ml of sterile distilled water, pelleted and resuspended

in sterile water to a final volume of 2.7 ml. To this 300 µl of 1M LiAc (pH 7.5) was added and incubated at 30°C for 15 min. The cells were mixed, divided into 500 µl aliquots, centrifuged for 30 sec at 14 000 rpm and each pellet resuspended in 0.1 M LiAc (pH 7.5) to a final volume of 250 µl. Glycerol was added to a final concentration of 15-20% and the cells were stored at -80°C.

**10. Yeast transformation**

Yeast cells were transformed using a modification of the protocol developed by Schiestl and Gietz (1989). All manipulations were performed using sterile technique. A 50 µl aliquot of competent cells (prepared as described above) were centrifuged at 14 000 rpm for 30 sec at room temperature. The supernatant was discarded and the following reagents were layered on the pellet: 240 µl 50% w/v PEG 4000, 36 µl 1 M LiAc (pH 7.5), 50 µg of herring sperm DNA and 5-10 µg of plasmid DNA or 20-50 µg of linear DNA and water to 350 µl. The pellet was resuspended with a pipette and the mixture was incubated at 30°C for 30 min, heat shocked at 42°C for 15 min and centrifuged at 14 000 rpm for 30 sec. The resulting pellet was resuspended in 400 µl of sterile water and 150 µl aliquots plated onto synthetic complete medium (Table 3) lacking the appropriate amino acids. The plates were incubated at 30°C for 3-5 days.

## 11. Complementation of the yeast *gim1* gene

Plasmids (no insert [p415-ADH], Yeast *GIM1* [pSG55], Mouse *GIM1* [pSG72], *Arabidopsis GIM1* [pJS19] and *Arabidopsis GIM1* with 6X His tag [pJS19H]) were transformed into SGY101 as described previously. Colonies showing growth on SC-leu plates (Table 3) were patched onto YPD medium (Table 3), grown overnight at 30°C and replica plated to YPD medium either with or without 2.5 μg/ml of benomyl and incubated at 14°C (7 days), 23°C (3 days) or 30°C (overnight).

## 12. Growth curves

The growth characteristics of transformed yeast cells were determined in two different ways: 1) Yeast cells were grown in SC-leu medium to stationary phase (2-3 days). An aliquot (2-4 ml) of this culture was inoculated into 100 ml of YPD containing benomyl (2.5 μg/ml) to give a starting $OD_{640}$ of 0.03-0.08 and the cells were incubated at various temperatures (14°C, 23°C and 30°C) with shaking at 225 rpm. The cells subsequently were allowed to double until they reached stationary phase (except for the cells grown at 14°C which were left until the positive control reached stationary phase). $OD_{640}$ readings were taken every 1-3 h for the 23°C and 30°C grown cells and every 8 h for the 14°C grown cells. 2) Yeast cells were grown to stationary phase and diluted into 100 ml YPD to an $OD_{640}$ of 0.015-0.025 and placed at 30°C with shaking at 225 rpm. These cells then were allowed to double until they reached early exponential phase ($OD_{640}$ ~0.08) at which point benomyl was added to a final concentration of 2.5 μg/ml

and the cells were transferred to 14°C and allowed to double until they reached stationary phase. $OD_{640}$ readings were taken every 1-5 h.

### 13. Yeast 2-hybrid

Competent HF7c and HF7c GIM5::TRP1 were prepared as described previously. Plasmids containing the inserts of interest (pACT14A, pACT19A and pACT108A) were each co-transformed with pAS2-ARACCA-Long into competent yeast HF7c and HF7c GIM5::TRP1 as described previously. Both the HF7c and HF7c GIM5::TRP1 transformants were plated on SC-leu-trp-his plates and a two-hybrid interaction was identified by the ability of the cells to grow in the absence of histidine. The cells were incubated at 30°C for 3-5 days.

### 14. Determination of *GIM1* expression in *Arabidopsis* tissue

To determine in which tissue the *Arabidopsis GIM1* gene was expressed, mRNA from rosette leaves, cauline leaves, floral stems and flowers was extracted (Cashmore *et al.*, 1980) and reverse transcribed by Dr. Diego Spertini (Concordia University) to generate cDNA. Equal amounts of this cDNA (1 μg) were used in PCR reactions as described previously.

**15. Protein expression and purification of *Arabidopsis* Gim1p**

The *E.coli* expression vector Plasmid pTric His containing the *Arabidopsis GIM1* gene was transformed into XL2-blue as described previously. A 5 ml YT-Amp (50 μg/ml) starter culture was inoculated with a single colony and incubated at 37°C overnight. The culture was transferred to 200 ml YT-Amp medium and incubated at 37°C until an $OD_{600}$ ~ 0.7 was reached at which point 250 μl of 0.8 M IPTG (isopropylthio-B-D-galactosidase) was added to induce protein expression. The culture was incubated for an additional 3 h and centrifuged at 4 500 rpm for 15 min at 4°C in a Beckman J2-HS JA-10 rotor. The pellet was resuspended in 15 ml of resuspension buffer (50 mM sodium phosphate [pH 7], 100 mM NaCl and 5 mM imidazole) to which 50 μl of protease inhibitor cocktail III (Calbiochem)/g *E. coli*, 0.15 mM PMSF and 1 ml lysozyme buffer (0.012 g lysozyme/ml resuspension buffer) was added. The sample was mixed, incubated at room temperature for 15 min, sonicated 10 times (10 sec pulse, 20 sec pause) on ice with a Sonifier 250 (Branson) and centrifuged at 9 500 rpm for 20 min at 4°C in a JA-20. The supernatant was applied to 5-10 ml of Talon CellThru Metal affinity resin (Clontech) (pre-equilibrated with 1X PBS), and incubated at 4°C for 30 min with gentle agitation. The slurry was placed in a 1 cm diameter column and the flow-through collected. The resin was washed with 60 ml of resuspension buffer and then the protein was eluted with 20 ml of elution buffer (50 mM sodium phosphate [pH 7], 300 mM NaCl and 150 mM imidazole). The resin was regenerated by washing with 25 ml of 20 mM MES buffer (pH 5) containing 0.1 M NaCl and stored at 4°C in 20% ethanol. The eluted protein was concentrated to an appropriate volume using the Millipore centrifugal filter

and buffer exchanged at 4°C in 20 mM Tris (pH 8), 150 mM NaCl using a 3 ml 10 000 MW Slidalyzer (Pierce). The sample concentration was determined using the Bradford Protein assay as recommended by the supplier (BioRad) and each sample was analyzed by SDS-PAGE (15%) for purity (Figure 8).

**16. Circular dichroism spectroscopy**

Secondary structure and thermal stability studies were conducted with the Jasco J-710 Spectropolarimeter, equipped with a thermostatically controlled cell holder. A CD cell of 200 µl volume and 0.05 cm path length was used. The *Arabidopsis* GIM1p was at a concentration of 0.75 mg/ml for analysis. Full spectra were recorded between 190 and 260 nm with the following settings: a scan speed of 50 nm/min, bandwith of 1.0 nm, response of 0.5 sec and a sensitivity of 100 mdeg. Two accumulations of scans were taken for the cell and buffer alone and five accumulations for the sample. The sample was also monitored as it was temperature denatured. The scans were done as described but the temperature was increased by 5°C increments from 25°C -90°C. The sample was allowed to equilibrate at each temperature for 5 min prior to scanning. The data were analyzed using the software supplied with the Jasco instrumentation and buffer and cell scans were subtracted from the sample. The melting temperature ($T_m$) was calculated using a plot of the denaturation curves at 222 nm. The percent α-helix was predicted using the secondary structure prediction program peptool lite version 1.1 from Twist tools (www.uib.no/People/mblpp/links_old/software.hym).

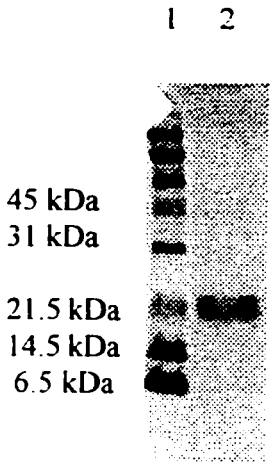



**Figure 8:** Polyacrylamide gel (15%) representing purified *Arabidopsis* Gim1 protein.

Lane 1: Broad range BioRad prestained SDS-PAGE standard
Lane 2: Purified *Arabidopsis* Gim1 protein

**17. Growth of *Arabidopsis***

*Arabidopsis* Col 0 seeds (20-50 µl) were incubated at 4°C in the dark for 2-3 days and then distributed to small pots. The plants were grown under 16 hour light and 8 hour dark for 2 weeks or until primary bolts emerged. Plants were watered every 2 days and given 20/20/20 (N/P/K) fertilizer (1 g/l) every 2 weeks. Primary bolts were cut to encourage proliferation of secondary bolts and the above ground part of the plants was dipped 4-6 days after clipping.

**18. Preparation of competent *Agrobacterium*** (Clough and Bent, 1998, modified from Bechtold *et al.*, 1993)

A single colony of *Agrobacterium* strain GV3101 was inoculated into 5 ml LB containing gentamycin (25 µg/ml) and incubated at 28°C for 2 days. The sample was divided into 350 µl aliquots and 50% glycerol was added to a final concentration of 15% prior to storing at -70°C. To prepare competent cells 200 ml of LB containing gentamycin (25 µg/ml) was inoculated with 100 µl of GV3101 freezer stock. The culture was incubated at 28°C until an $OD_{600}$ of 0.6-0.9 was reached. The cells were centrifuged at 3000 rpm (JA-14) for 10 min at 4°C. The pellet was resuspended in 1 ml of sterile 20 mM $CaCl_2$, placed in liquid nitrogen for 10 sec and then stored at -70°C.

## 19. *Agrobacterium* and *Arabidopsis* Transformation

Plasmid DNA (0.5-3 μg) was added to a 50 μl aliquot of competent GV3101. The sample was frozen in liquid nitrogen for 1 min, thawed at 37°C for 5 min and then 500 μl of LB and gentamycin (25 μg/ml) was added with incubation at 28°C for 3.5 hrs. Aliquots (250 μl) of the transformed cells were plated on minimal AB medium (Table 3) containing gentamycin (25 μg/ml) and kanamycin (50 μg/ml) and incubated at 28°C for 2-4 days.

Long day grown Col 0 *Arabidopsis* plants were used for *Agrobacterium* transformation. A small liquid culture (10 ml) of minimal AB containing 25 μg/ml gentamycin and 50 μg/ml kanamycin was inoculated with *Agrobacterium* GV3101 carrying the plasmid of interest and incubated at 28°C until stationary phase. This was then transferred to a large liquid culture (500 ml) and incubated once again at 28°C until stationary phase was reached. The *Agrobacterium* was centrifuged at 7000 rpm (JA-14) and the pellet resuspended to an $OD_{600}$ of 0.8 in fresh 5% sucrose. Silwett L-77 was added to a concentration of 0.05%. The *Agrobacterium* solution was poured into a plastic container and the above ground part of the plant was dipped for 2-5 sec. The dipped plants were covered with plastic wrap and grown normally. When the plants became mature they were set aside and allowed to dry. The dry seeds from each transformed line were harvested and collected in an Eppendorf tube and stored in the dark at room temperature.

**20. Selection of transformants**

Seeds (20 µl) were placed in a sterile Eppendorf tube to which 1 ml of sterilization solution (1.05% sodium hypochlorite and 0.02% SDS) was added and incubated at room temperature with gentle mixing for 10 min. The seeds were allowed to settle, washed 5 times with sterile water and incubated at 4°C in the dark for 3 days. Transformants were selected by plating the seeds on MS selection medium containing 50 µg/ml kanamycin. The green plants were retained while yellow ones were discarded (5-7 days). The transformed plants were placed in soil and grown to seed under normal long day conditions.

**21. Localization in tobacco protoplasts**

All solutions for this procedure were sterile and each step was done aseptically in a laminar flow hood. Four week old tobacco plants were used for protoplast formation. Leaves were harvested and the upper surface gently brushed on Whatman paper with carborundum (silicon carbide) using a fine paintbrush. This removes the fine hairs, cuticle and trichomes. The leaves were rinsed twice by gentle agitation in a beaker containing 250 ml of water, patted dry on Whatman filter paper to remove excess water, placed in a glass Perti dish containing 15-20 ml of plasmolysis solution (Table 3) and incubated in the dark for 4-6 h. The solution was gently removed with a syringe and 15-20 ml enzymatic solution (Table 3) was added with incubation overnight in the dark. The enzymatic solution was removed with a syringe without disturbing the leaves and 15 ml

of dense solution (Table 3) was added. The leaves were shaken gently with metal forceps to liberate the protoplasts. The digested leaves are filtered through cheesecloth and collected in a sterile Babcock bottle. The volume of the filtrate in the bottle was adjusted to 4 ml with dense solution and then the bottle was filled with culture medium (Table 3). The samples were centrifuged at low speed for 10 min and the protoplast interphase layer collected and placed on ice. The protoplasts were counted on a hemocytometer and diluted to a final concentration of 1 million cells/ml. Endofree DNA (40 µl of 1 µg/ul) was aliquoted into an Eppendorf tube, mixed with an equal volume of 2X electroporation buffer (Table 3) and 320 µl of protoplasts. The contents of the tube were mixed gently and transferred with a pipette to an electroporation cuvette (1 $cm^2$). The samples were electroporated at 225 V, 1000 µF and immediately placed on ice for 10 min (Matton *et al.*, 1993). The electroporated protoplasts were transferred to 3.5 ml of culture medium (Table 3) in a 60 mm Petri dish and incubated in the dark for 24 h. Slides were prepared a few minutes prior to use. An aliquot (20 µl) of protoplasts was placed on a slide, covered with a cover slip and then sealed with clear nail polish. The cover slip was sealed to prevent evaporation and because a Leica DMIRBE confocal inverted microscope with the DD488/568 filter set was used to view the protoplasts. A pinhole size of 1.0 and the 63X objective was used. The following beam path settings were used: pMT1 for phase contrast, pMT2 with a capture wavelength between 500-530 nm (green) for the GFP fluorophore, pMT3 with a capture wavelength between 670-700 nm (red) for the chlorophyll and a signal gain set at 1020 V (variable). Samples were photographed in green, red and phase contrast and then overlayed.

## Results

### 1. Isolation of the *Arabidopsis* GIM1 homologue

Utilizing the yeast 2-hybrid system with tRNA nucleotidyltransferase as bait a cDNA coding for the *Arabidopsis thaliana* homologue of the yeast *GIM1* gene product was isolated by Jun Gu in our lab. The identity of this gene product was first predicted based on its similarity to the yeast Gim1p (Figure 6). Structural data or functional complementation of a yeast *Gim1* mutant would help to more firmly establish the identity of the *Arabidopsis* protein.

### 2. Structural analysis of Gim1p

To provide evidence that the α–helical coiled coil regions that are present in archaeal Gim α and β proteins (Leroux *et al.*, 1999) are also present in the *Arabidopsis* Gim1p, circular dichroism (CD) was performed on the *Arabidopsis* Gim1p. The spectra obtained are typical of proteins containing primarily α–helices. There is a distinct trough at 222 and at 209 nm. (Figure 9). Overall the CD spectrum is similar to Gim1p from other organisms (Leroux *et al.*, 1999). The sample was also temperature denatured in 5°C increments from 25°C to 90°C. The decrease in intensity of the two troughs at 222 and 209 nm (Figure 9) indicates that the protein unfolded gradually with no intermediate forms. The relative intensity of the trough at 222 nm was plotted to demonstrate gradual unfolding with no plateau (Figure 10). The $T_m$ for the unfolding of the *Arabidopsis* Gim1p was calculated to be 42°C.

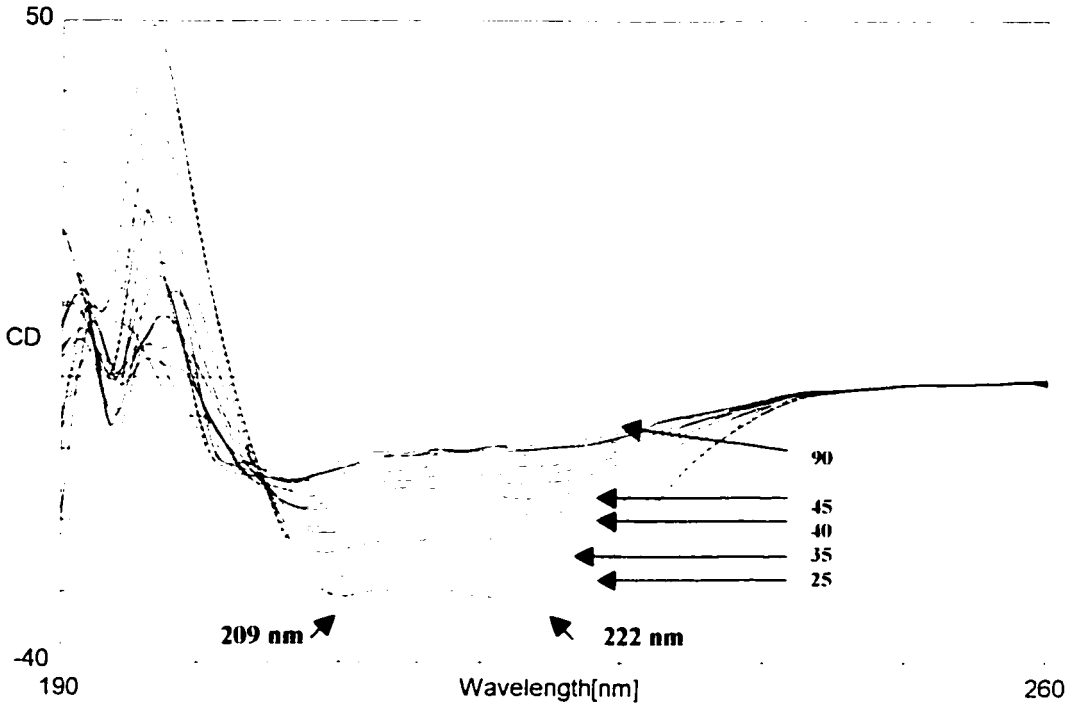



**Figure 9:** Circular dichroism spectra of the *Arabidopsis* Gim1p taken at 5° increments between 25 °C and 90 °C.

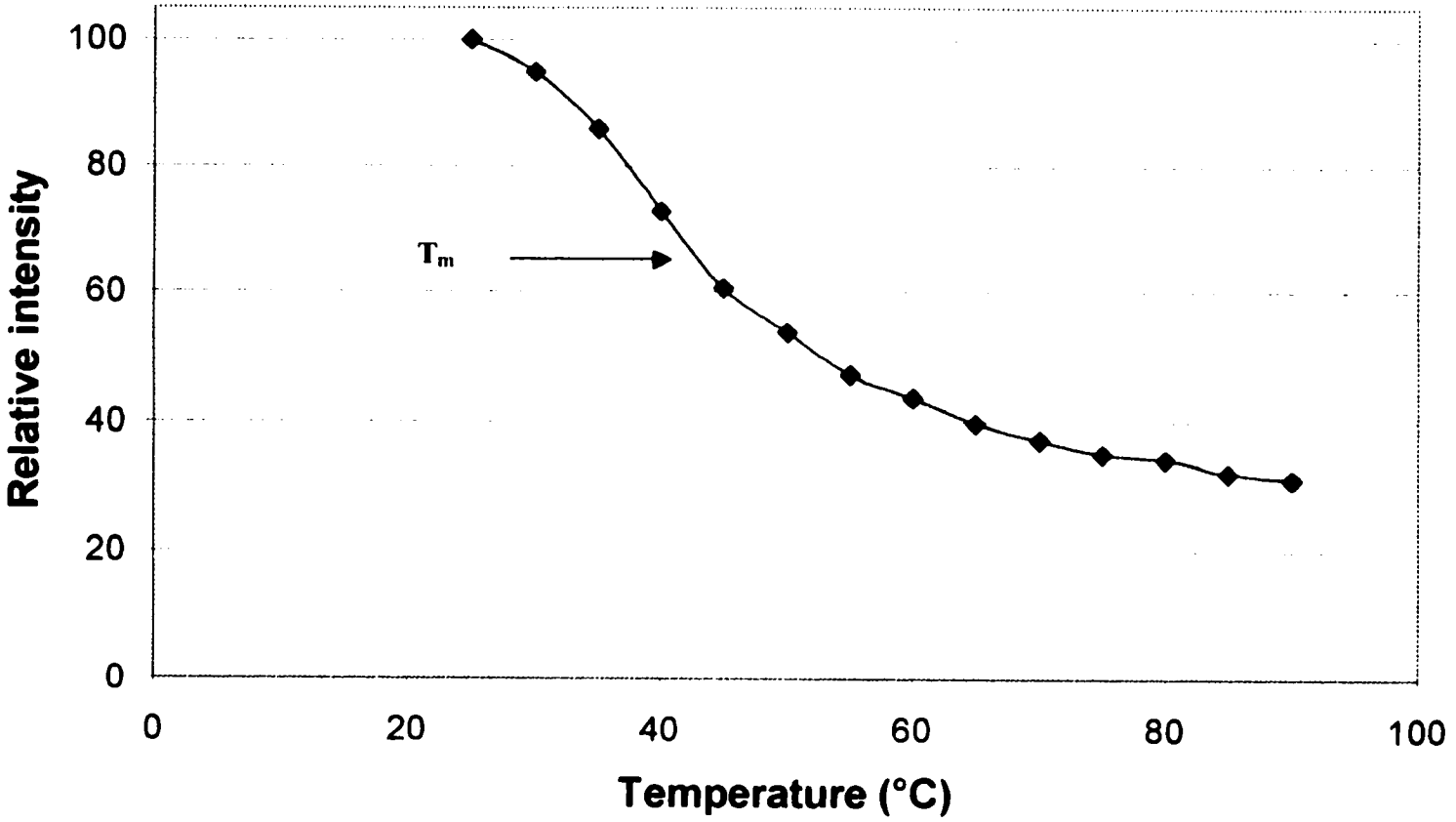



**Figure 10**: Plot of the relative CD intensity at 222 nm versus temperature. The highest CD value (25°C ) is designated 100% and all values are adjusted accordingly. The arrow indicates the calculated $T_m$ value of 42°C.

**3. Complementation analysis**

The open reading frame from the *Arabidopsis GIM1* cDNA was PCR amplified from the yeast two-hybrid vector (pACT19A) and fragments corresponding to the expected size of the open reading frame with (436 bp) and without (416 bp) an added C-terminal 6X His tag were generated (Figure 11). The fragments were digested with the appropriate restriction enzymes and cloned into p415-ADH to generate pJS19 (*Arabidopsis GIM1*) and pJS19H (*Arabidopsis GIM1* with 6X His tag). Restriction digestion confirmed single insertion events and the correct orientation of the inserts (Figure 12). Subsequently, these plasmids were transformed into the *Gim1::KanMX4* disrupted yeast strain, SGY101. Cells were plated on SC-leu medium and colonies showing leucine prototrophy were patched to SC-leu plates, grown overnight, replica plated to YPD medium and incubated at three different temperatures (14°C, 23°C or 30°C). In addition to the plasmids containing the *Arabidopsis* cDNAs, SGY101 also was transformed with p415-ADH alone (negative control) or p415-ADH containing the yeast *GIM1* gene or mouse *GIM1* cDNA (positive controls). All of the strains appeared to grow well at all of the temperatures tested although the p415-ADH negative control may have grown slightly less well at 14°C (Figure 13). To intensify any differences in growth rate the experiment was repeated in the presence of 2.5 μg/ml benomyl. Under these conditions there is a significant decrease in yeast cell growth for p415-ADH (no insert) alone at 14°C and at 23°C. Equally as significant, there is no obvious difference in growth in the strains with the *GIM1* genes from mouse, yeast or *Arabidopsis thaliana*

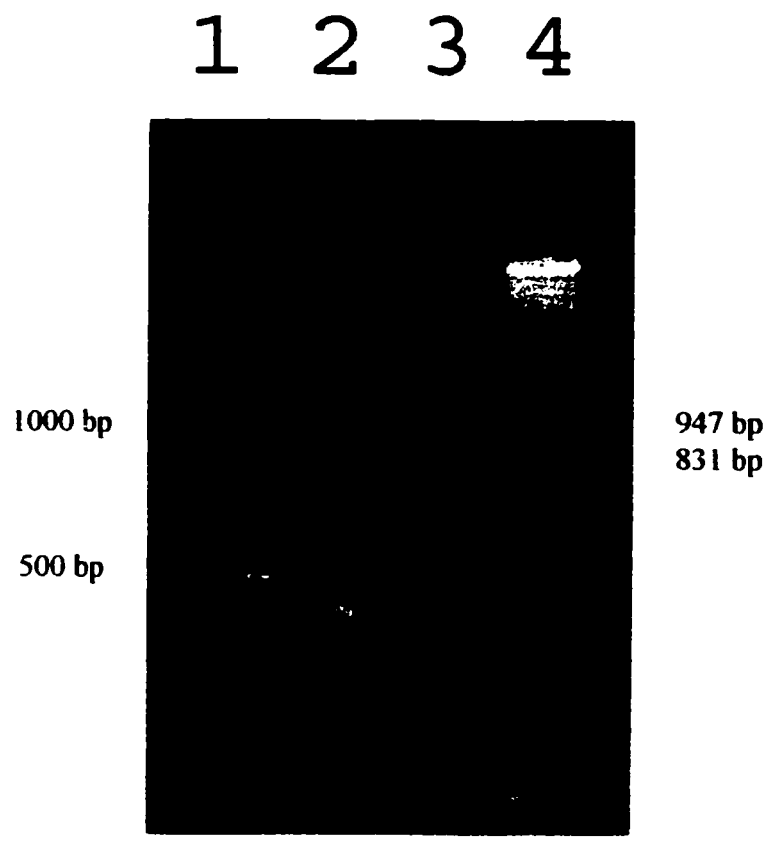



**Figure 11:** Agarose gel showing products generated from PCR amplification of the *Arabidopsis GIM1* open reading frame.

Lane 1: 100 bp DNA marker
Lane 2: PCR product of the *Arabidopsis GIM1* gene
Lane 3: PCR product of the *Arabidopsis GIM1* gene containing 6X His tag
Lane 4: *Eco*RI-*Hin*dIII lambda marker

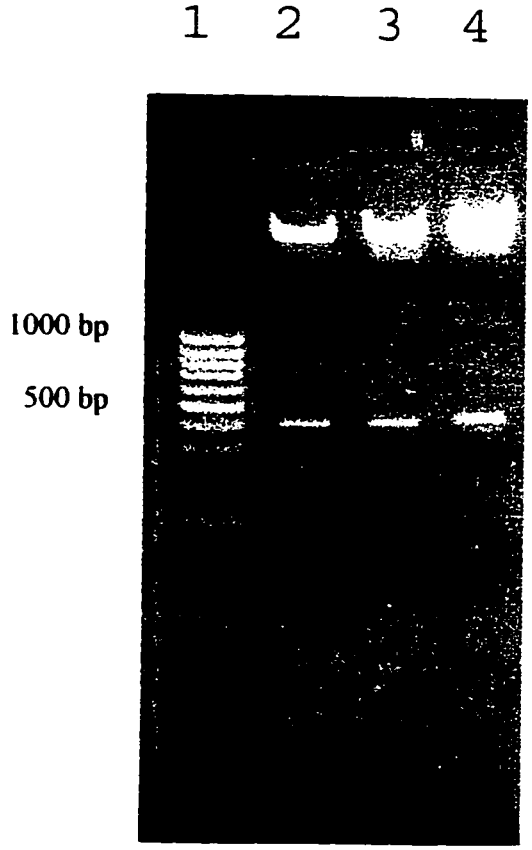



**Figure 12:** Agarose gel showing restriction digestion of p415-ADH containing the *Arabidopsis GIM1* gene. The plasmids were digested with *Hin*dIII and *Bam*HI

Lane 1: 100 bp DNA marker
Lane 2 and 3: p415-ADH containing the *Arabidopsis GIM1* in the correct orientation
Lane 4: p415-ADH containing the *Arabidopsis GIM1* with the His tag in the correct orientation

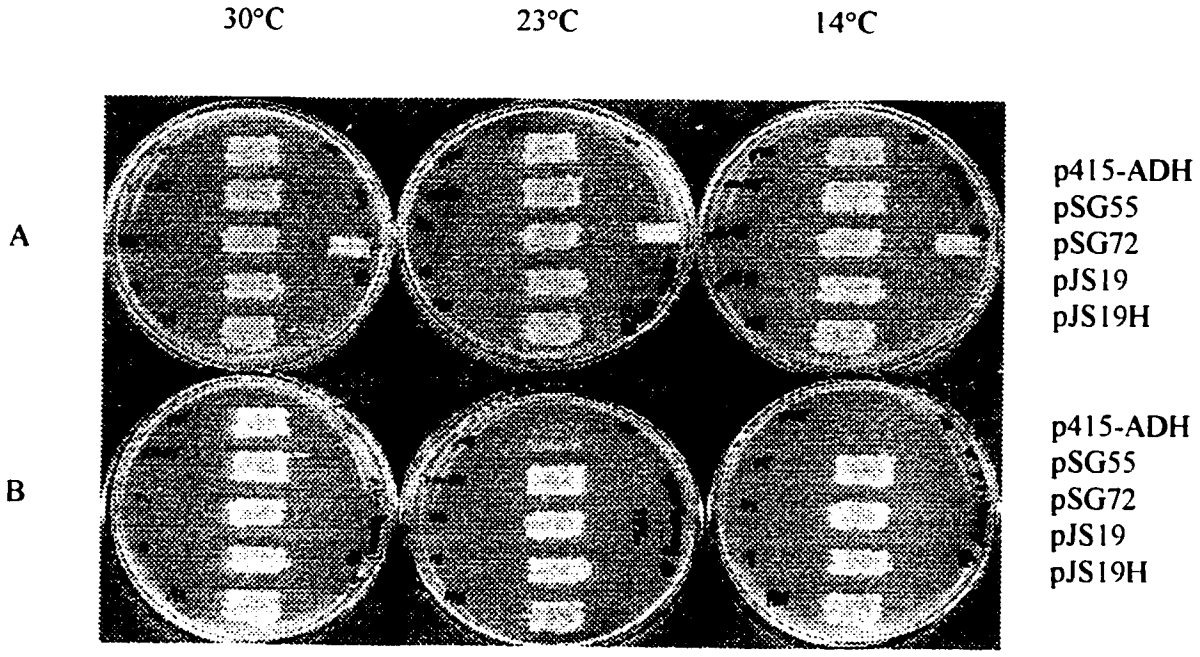



**Figure 13:** Replica plating of SGY101 containing the following plasmids:
p415-ADH – plasmid alone
pSG55-yeast *GIM1* gene in p415-ADH
pSG72-mouse *GIM1* cDNA in p415-ADH
pJS19-*Arabidopsis GIM1* cDNA in p415-ADH
pJS19H-*Arabidopsis GIM1* cDNA with 6X His tag in p415-ADH

(A) YPD medium at temperature indicated
(B) YPD medium containing 2.5 μg/ml benomyl at temperature indicated

(Figure 13). Growth curves were generated to more precisely determine the ability of the *Arabidopsis* cDNA to complement the yeast growth defect.

**4. Growth curves**

As no differences were seen in the replica plating results in the absence of 2.5 μg/ml benomyl, growth curves were calculated only for samples in the presence of benomyl. Growth curves were generated under two different experimental conditions and the differences in growth rates were analyzed. All experiments at 14°C or 30°C were done on two separate occasions. When the cells were allowed to grow to stationary phase, diluted into 100 ml of YPD medium containing benomyl to an $OD_{640}$ of 0.03 and then incubated at 30°C there was no significant difference in growth rate among any of the transformed strains (Figure 14). On the other hand, when the cells were treated the same way but then incubated at 23°C there was a difference in growth pattern in that the strain carrying p415-ADH (no insert) alone grew more slowly than the strains carrying either the yeast or *Arabidopsis GIM1* genes (Figure 15). This effect was even more dramatic in the yeast cells that were grown at 14°C (Figure 16).

For the second set of growth curves the cells were allowed to grow to stationary phase, diluted in 100 ml YPD to an $OD_{640}$ of 0.02 and allowed to grow until the $OD_{640}$ had reached 0.08 (early exponential phase ~11-12 hrs) at which time benomyl was added and the temperature was shifted to 14°C. Figure 17 indicates that the addition of benomyl and the temperature shift to 14°C reduces the doubling time of the strain

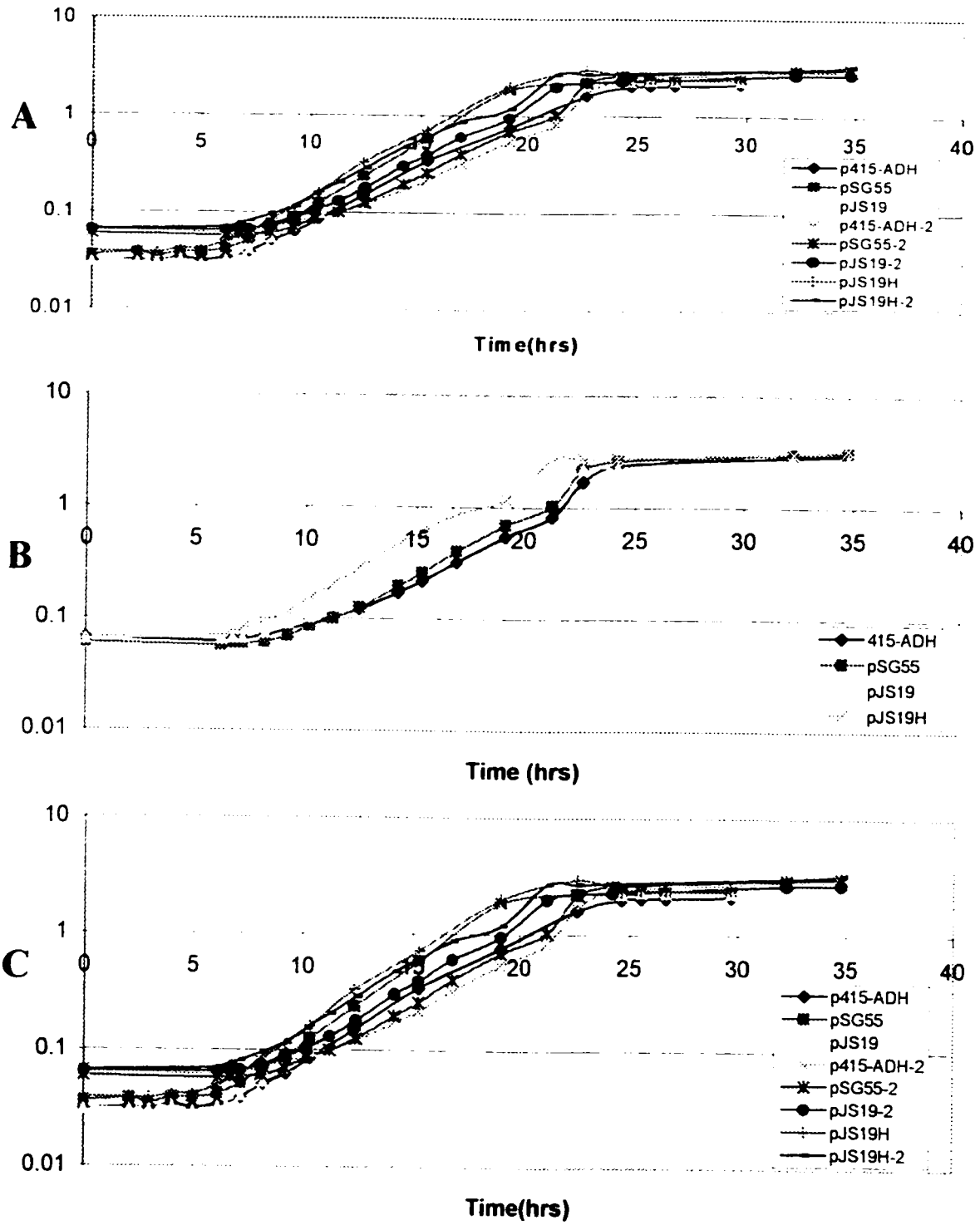



**Figure 14:** Growth curves for growth at 30°C in YPD containing benomyl (2.5 μg/ml). A stationary phase culture of SGY101 containing the plasmid indicated was diluted into fresh YPD containing 2.5 μg/ml of benomyl and incubated at 30°C for the time indicated. A and B represent the same experiment conducted on separate days. C is a combination of all the data. The plasmids are: p415-ADH (no insert), pSG55 (yeast *GIM1*), pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag).

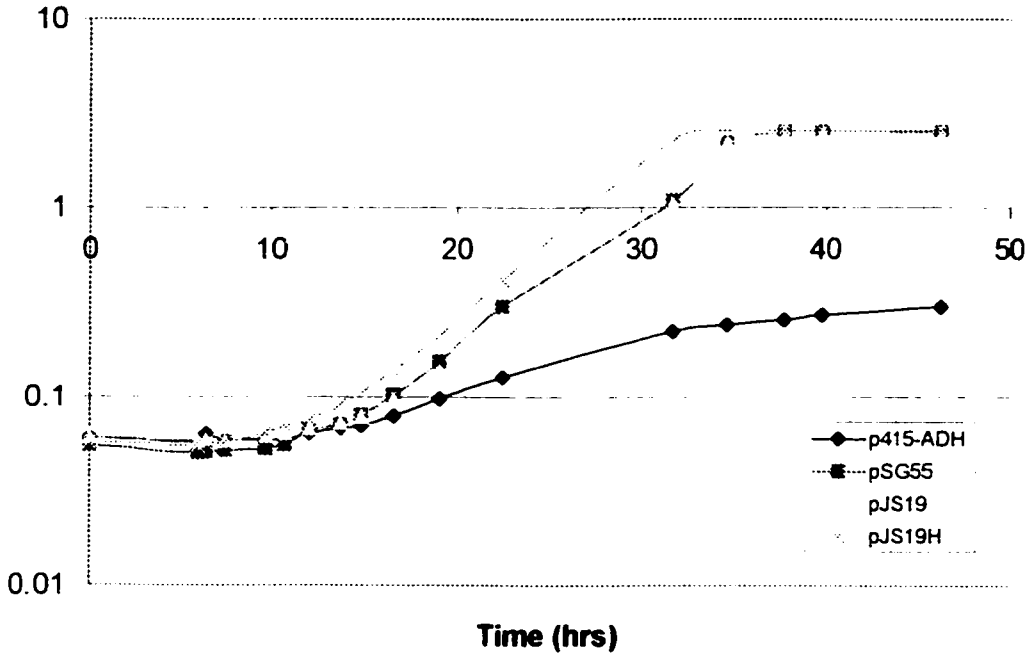



**Figure 15**: Growth curves for growth at 23°C in YPD containing 2.5 μg/ml benomyl. A stationary phase culture of SGY101 carrying the indicated plasmid was diluted into fresh YPD containing benomyl (2.5 μg/ml) and incubated at 23°C for the time indicated. The plasmids are: p415-ADH (no insert), pSG55 (yeast *GIM1*), pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag).

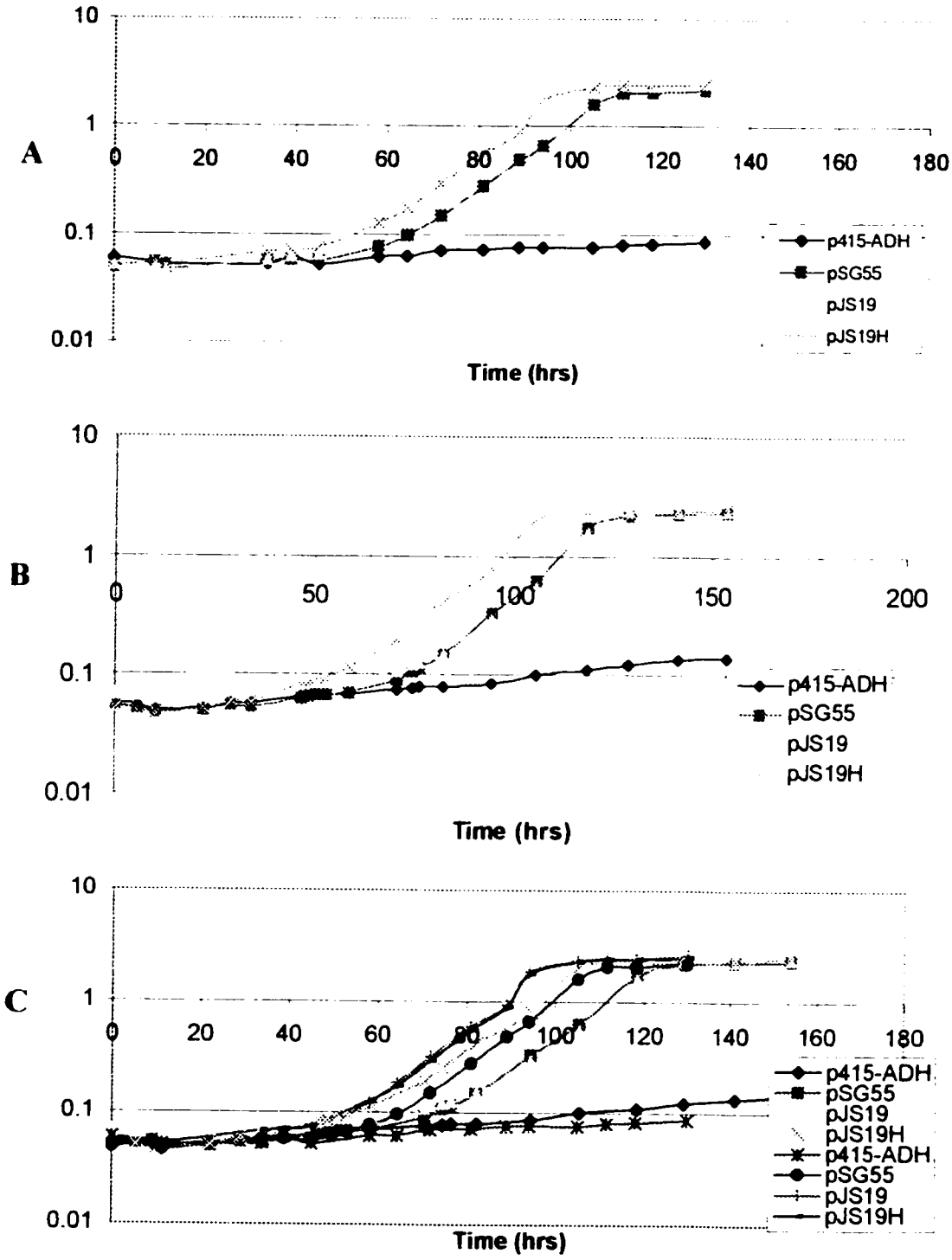



**Figure 16:** Growth curves for growth at 14°C in YPD containing 2.5 µg/ml benomyl. A stationary phase culture of SGY101 carrying the indicated plasmid was diluted into fresh YPD containing benomyl (2.5 µg/ml) and incubated at 14°C for the time indicated.
A and B represent the same experiment conducted on separate days. C is a combination of all the data. The plasmids are: p415-ADH (no insert), pSG55 (yeast *GIM1*), pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag).

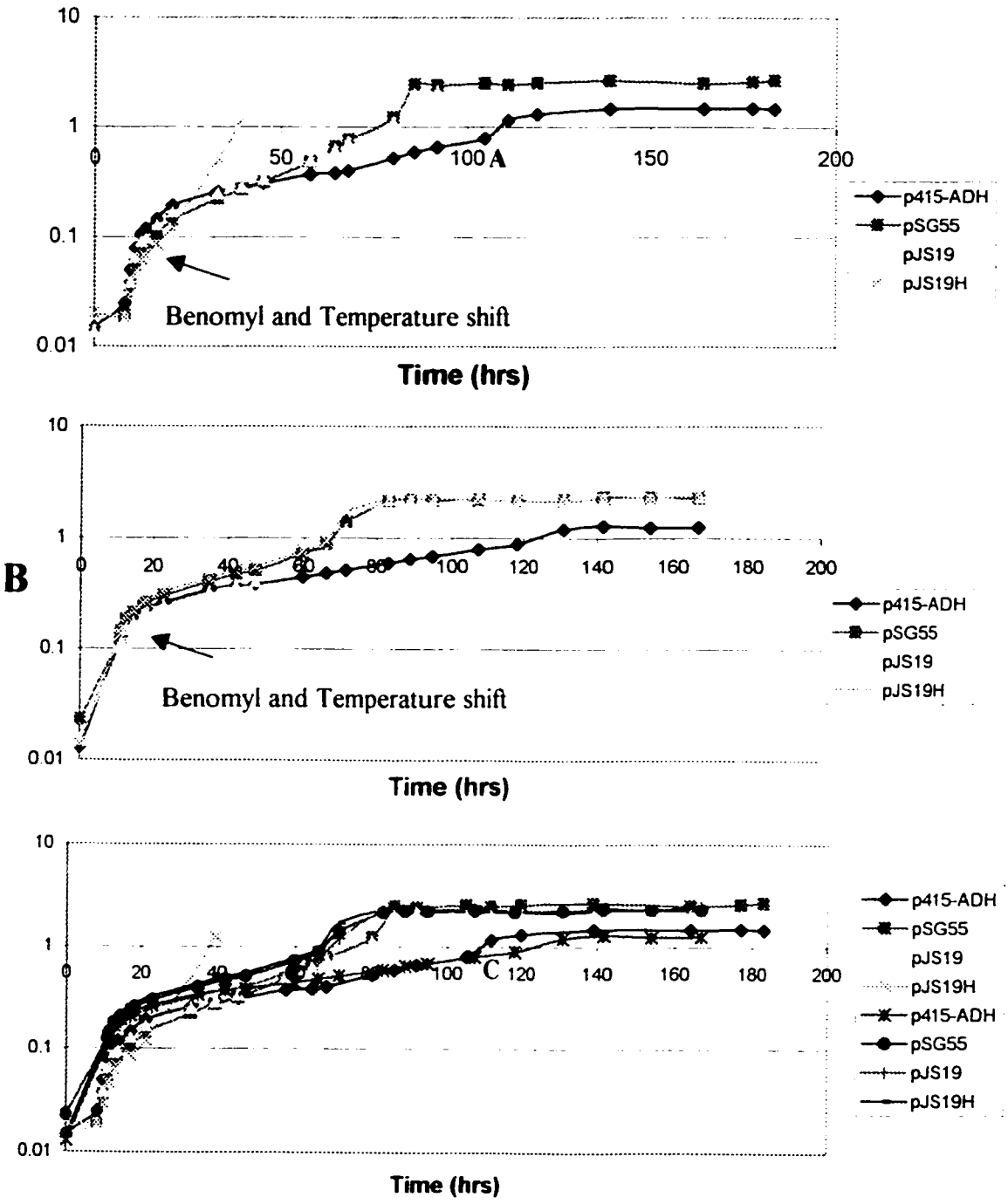



**Figure 17**: Growth curves for exponentially growing SGY101 cells containing the plasmid indicated to which benomyl (2.5 μg/ml) was added and the temperature shifted to 14°C. The arrow represents the time at which benomyl was added and the temperature shifted. A and B represent the same experiment conducted on separate days. C is a combination for all the data at the times indicated. The sample containing pJS19H in experiment A was terminated due to contamination. The plasmids are: p415-ADH (no insert), pSG55 (yeast *GIM1*), pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag).

carrying p415-ADH (no insert) over the time course of the experiment. In contrast, there is no difference between the *Arabidopsis* and yeast *GIM1* strains (Figure 17).

**5. Disruption of GIM5**

Gim1p is part of GIMC (Genes Involved in Microtubule Biogenesis Complex) in yeast. To address the question of whether this complex is needed for interaction with tRNA nucleotidyltransferase, the *GIM5* gene (coding for another constituent of this complex) of the yeast strain HF7c was disrupted with *TRP1*. The *TRP1* gene was PCR amplified from pRS314 and transformed into competent HF7c. After transformation of HF7c with this DNA fragment containing the yeast *TRP*1 gene and 50 bp of *GIM5* sequence at each end, 15 potential candidates were isolated on SC-Trp plates. The cells were patched on new SC-Trp plates and then replica plated onto YPD plates containing benomyl and grown at 14°C and 30°C. Two colonies demonstrated cold sensitivity (data not shown), indicating disruption of one of the GIM genes. The location of the DNA insertion in the potentially disrupted strains was confirmed by yeast colony PCR (Figure 18). Products of ~ 650 bp (lane 2 and 3) and ~ 1.2 kbp (lane 4 and 5) corresponding to *GIM5* (control) and *GIM5::TRP*1, respectively, were generated.

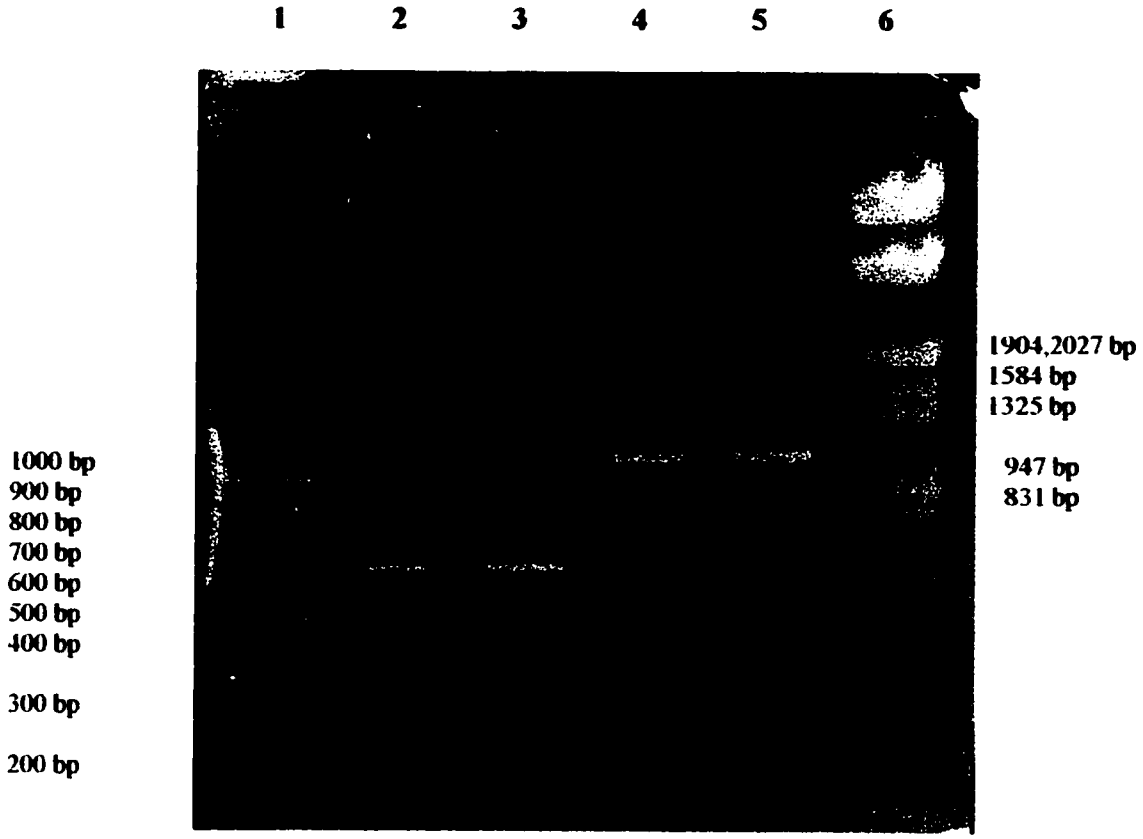



**Figure 18:** Agarose gel of the PCR products of colony PCR of HF7c and HF7c GIM5::TRP1.

Lane 1: 100 bp DNA marker
Lane 2,3: PCR product of HF7c
Lane 4,5: PCR product of HF7c GIM5::TRP1
Lane 6: *Eco*RI-*Hind*III lambda marker

## 6. Interaction of *Arabidopsis GIM1* with tRNA nucleotidyltransferase in HF7c Gim5::TRP1

Yeast strain HF7c GIM5::TRP1 was used to test the ability of *Arabidopsis* Gim1p to interact with tRNA nucleotidyltransferase in a yeast two-hybrid assay. Plasmids pAS2-ARACCA-Long and pACT19 (*Arabidopsis GIM1*) were co-transformed into the wild-type and disrupted strains and plated on SC-leu-trp-his plates. Also, plasmids pAS2-ARACCA-Long and pACT14A (positive control) and plasmids pAS2-ARACCA-Long and pACT108A (negative control) were transformed into these strains. The expected results were obtained from the HF7c yeast strain, that is, pACT14A and pAS-ARACCA-Long showed an interaction (~ 150 colonies) as did pACT19A and pAS2-ARACCA-Long (160 colonies). As expected the negative control plasmid pair (pACT108A and pAS2-ARACCA-Long) showed no interaction (Table 7). In the disrupted yeast strain (HF7c GIM5::TRP1) pACT108A and pAS2-ARACCA-Long again showed no interaction while pACT14A and pAS2-ARACCA-Long showed the expected interaction. In contrast, the plasmid pair pACT19A and pAS2-ARACCA-Long now showed no interaction (Table 7).

## 7. Identification of *Arabidopsis GIM1* mRNA in *Arabidopsis thaliana* tissue

Genomic *Arabidopsis* DNA was PCR amplified with oligonucleotides specific for the *Arabidopsis GIM1* gene or cDNA. The reaction generated a fragment of ~700 bp when *Arabidopsis* chromosomal DNA was used as a template (Figure 19A). When cDNA from

**Table 7: Yeast two-hybrid results**

Screening for an interaction with pAS2-ARACCA-Long (Bait)

| Plasmid (Prey) | Strain | |
|---|---|---|
| | HF7c | HF7c GIM5::TRP1 |
| pACT14A (+ control) | 150 colonies | 116 colonies |
| pACT19A (*Arabidopsis GIM1*) | 160 colonies | 2 colonies |
| pACT108A (- control) | 0 colonies | 0 colonies |

Legend: A positive interaction between the bait and the prey plasmids was defined by growth on $His^-$ plates.

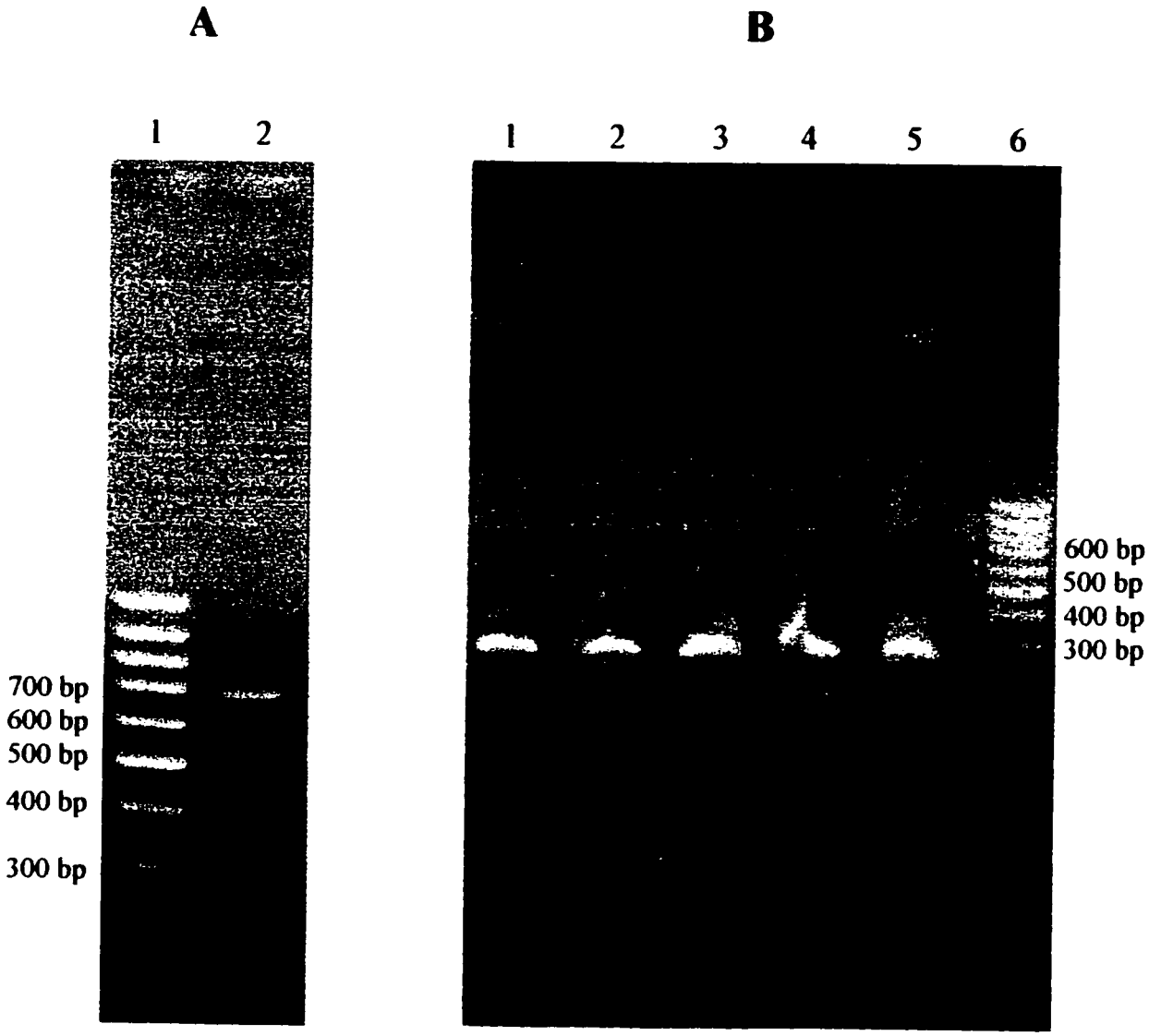



**Figure 19:** Agarose gel of PCR products generated from *Arabidopsis* genomic DNA and cDNA using GIM1 specific primers

(A) Agarose gel of PCR products generated from *Arabidopsis* genomic DNA
Lane 1: 100 bp DNA marker
Lane 2: ~ 700 bp fragment representing *GIM1* PCR product.

(B) Agarose gel of PCR products generated from *Arabidopsis* cDNA isolated from the tissue indicated.
Lane 1: cauline leaves
Lane 2: rosette leaves
Lane 3: floral leaves
Lane 4: flowers
Lane 5: pJS19 control
Lane 6: 100 bp DNA marker

various *Arabidopsis* tissues were used as template with the same oligonucleotides, products of the expected size (~300 bp) were generated (Figure 19B).

## 8. Phenotypic analysis of *Arabidopsis thaliana* tRNA nucleotidyltransferase overexpression in *Arabidopsis*

The region of the *Arabidopsis thaliana* cDNA between ATG1 and ATG3 containing the predicted mitochondrial and/or chloroplast targeting signal was PCR amplified from pBIN35SmGFP4-ARACCA-Long (*Arabidopsis* tRNA nucleotidyltransferase cDNA containing the targeting signal). A PCR product of the expected size (250 bp) was generated (Figure 20). The fragment was digested with the appropriate restriction enzyme and cloned into pBIN35SmGFP4 to generate pBIN35SmGFP4-ARACCA-TS (*Arabidopsis* targeting signal). Restriction digestion confirmed a single insertion event and the correct orientation (Figure 21). A fragment of expected length was evident in lanes 3, 5 and 6 while lanes 2 and 4 contain an insert in the reverse orientation. Plasmids pBIN35SmGFP4-ARACCA-Long (*Arabidopsis* tRNA nucleotidyltransferase containing the targeting signal), pBIN35SmGFP4-ARACCA-Short (*Arabidopsis* tRNA nucleotidyltransferase lacking the targeting signal) and pBIN35SmGFP4-ARACCA-TS (*Arabidopsis* targeting signal) were transformed into *Agrobacterium tumefaciens* GV3101, as described previously and plated on MS medium (Table 3) containing 50 μg/ml kanamycin and 25 μg/ml gentamycin for selection. For each transformation 2-5 colonies were obtained. Cells from these colonies were inoculated into minimal AB medium (Table 3) containing the appropriate antibiotics and prepared for transformation

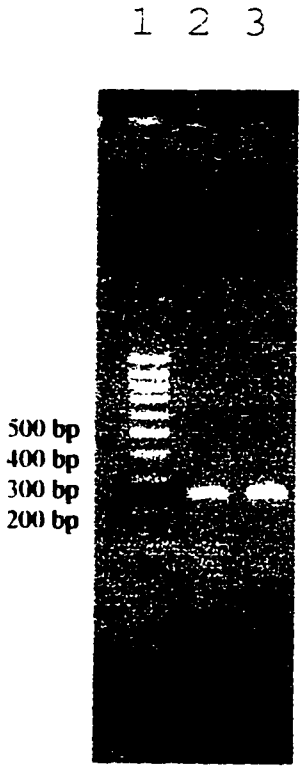



**Figure 20:** Agarose gel of PCR product generated from *Arabidopsis* cDNA using primers to amplify region between ATG1 and ATG3.

Lane 1: 100 bp DNA marker
Lane 2,3: PCR product generated representing potential organellar targeting sequence

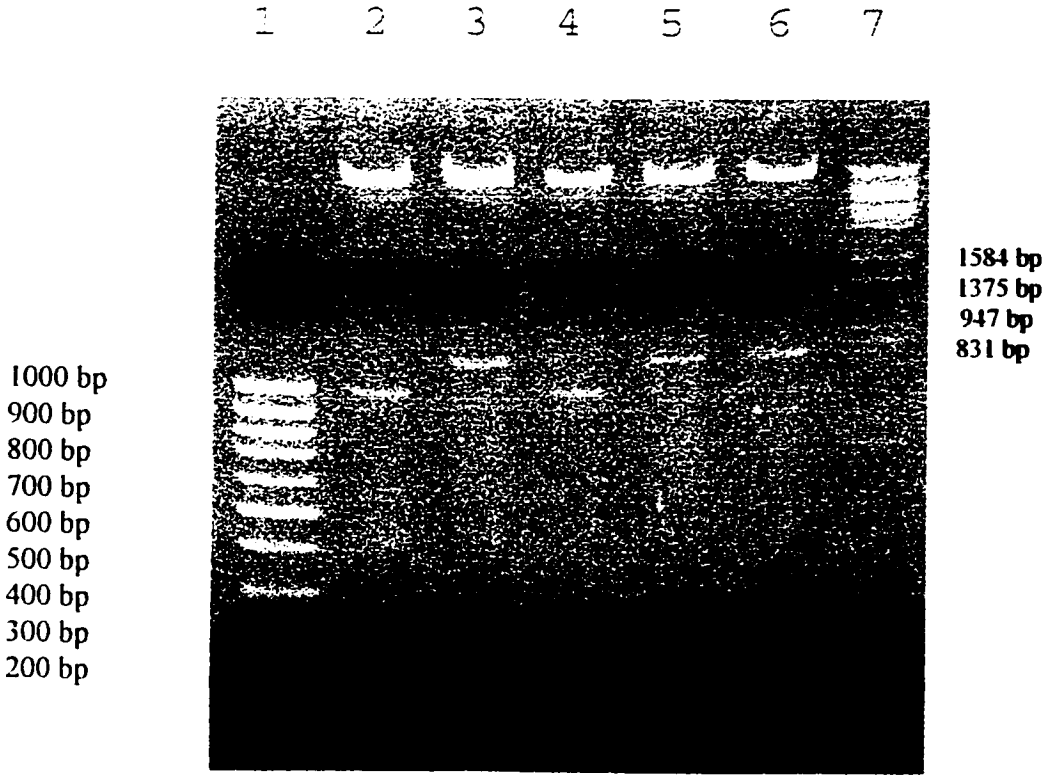



**Figure 21:** Agarose gel of restriction digestions of pBIN35SmGFP4-ARACCA-TS with *Sac*I

Lane 1: 100 bp DNA marker
Lane 2,4: pBIN35SmGFP4 containing the *Arabidopsis* targeting signal in the reversed orientation
Lane 3,5,6: pBIN35SmGFP4 containing the *Arabidopsis* targeting signal in the correct orientation
Lane 7: *Eco*RI-*Hind*III lambda marker

as described previously. Plants were dipped, transferred back to pots and allowed to grow to seed. The seeds for each construct were collected, sterilized and selected on MS selection medium containing kanamycin. After the first selection less than 1% of the seeds were green indicating an insertion event (Table 8). The green plants were self-crossed, grown once again to seed, the seeds collected, germinated and screened once again on MS medium containing kanamycin. Plants were analyzed for any phenotypic abnormalities on a weekly basis until they were mature. In all stages there were no physically detectable phenotypes.

**9. Localization of tRNA nucleotidyltransferase in *Arabidopsis thaliana***

The *Arabidopsis* tRNA nucleotidyltransferase containing the potential targeting signal (CCA-Long), lacking the targeting signal (CCA-Short) or the targeting signal alone (CCA-TS) were amplified by PCR from pAS2-ARACCA-Long. The expected fragments of ~1950 bp, ~1700 bp and ~250 bp correspond to ARACCA-Long, ARACCA-Short and ARACCA-TS, respectively were generated (Figure 22). The PCR fragments were digested with the appropriate restriction enzyme and cloned into pBIN35S35SEmGFP to generate pBIN35S35SEmGFP-ARACCA-Long (*Arabidopsis* tRNA nucleotidyltransferase containing the targeting signal) and pBIN35S35SEmGFP-ARACCA-Short (*Arabidopsis* tRNA nucleotidyltransferase lacking the targeting signal). Restriction digests once again confirmed single insertion events and correct orientation (Figure 23). Plasmids showing the expected restriction pattern were transformed into XL2-Blue and extracted from the cells using the EndoFree Kit as described previously. The DNA was transformed into tobacco protoplasts as described and viewed on a Leica

**Table 8: *Arabidopsis* transformation results**

| | ARACCA-TS | | ARACCA-Short | | ARACCA-Long |
|---|---|---|---|---|---|
| Total plants transformed ($F_0$) | 20 | | 20 | | 20 |
| Seeds selected | >15000 | | >10000 | | >10000 |
| Green seedlings ($F_1$) | 9/10000 | | 3/8000 | | 5/12000 |
| True transformants | 2 | | 2 | | 1 |
| | Plant #1 | Plant #2 | Plant #1 | Plant #2 | Plant #1 |
| Ratio of green to yellow seeds | 1:1 | 97:3 | 470:40 | 500:30 | 300:0 |
| Percentage green ($F_2$) | 50% | 97% | 92% | 94% | 100% |
| $F_3$ generation | 100 % green | | 100 % green | | 100 % green |

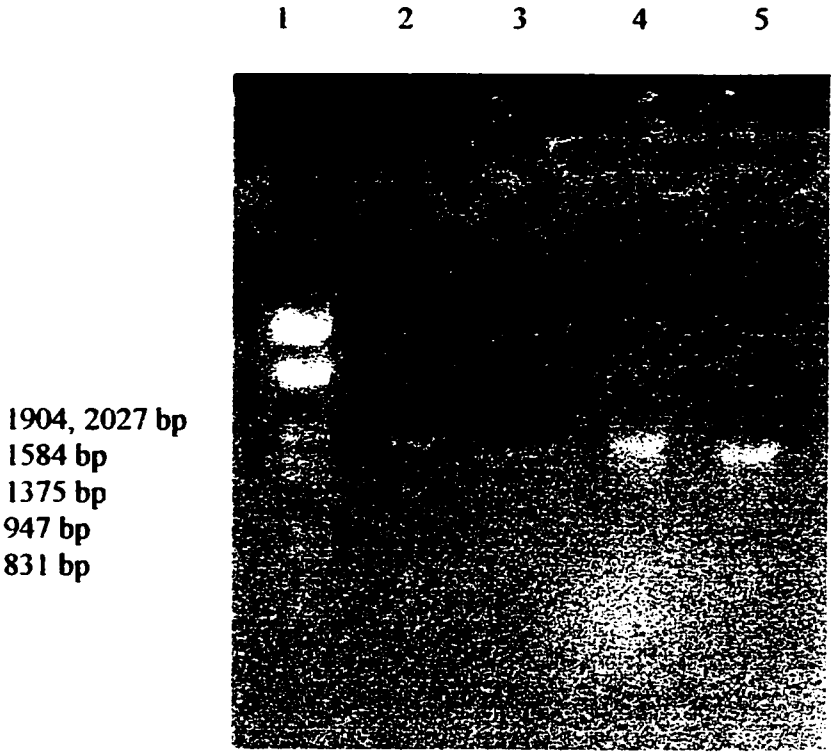



**Figure 22:** Agarose gel showing products generated from PCR amplification of the *Arabidopsis* tRNA nucleotidyltransferase cDNA

Lane 1: *Eco*RI-*Hind*III lambda marker
Lane 2: PCR product of the *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG1 (~ 1895 bp)
Lane 3: PCR product of the *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG3 (~ 1700 bp)
Lane 4: PCR product of the *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG1 (~ 1895 bp)
Lane 5: PCR product of the *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG3 (~ 1700 bp)

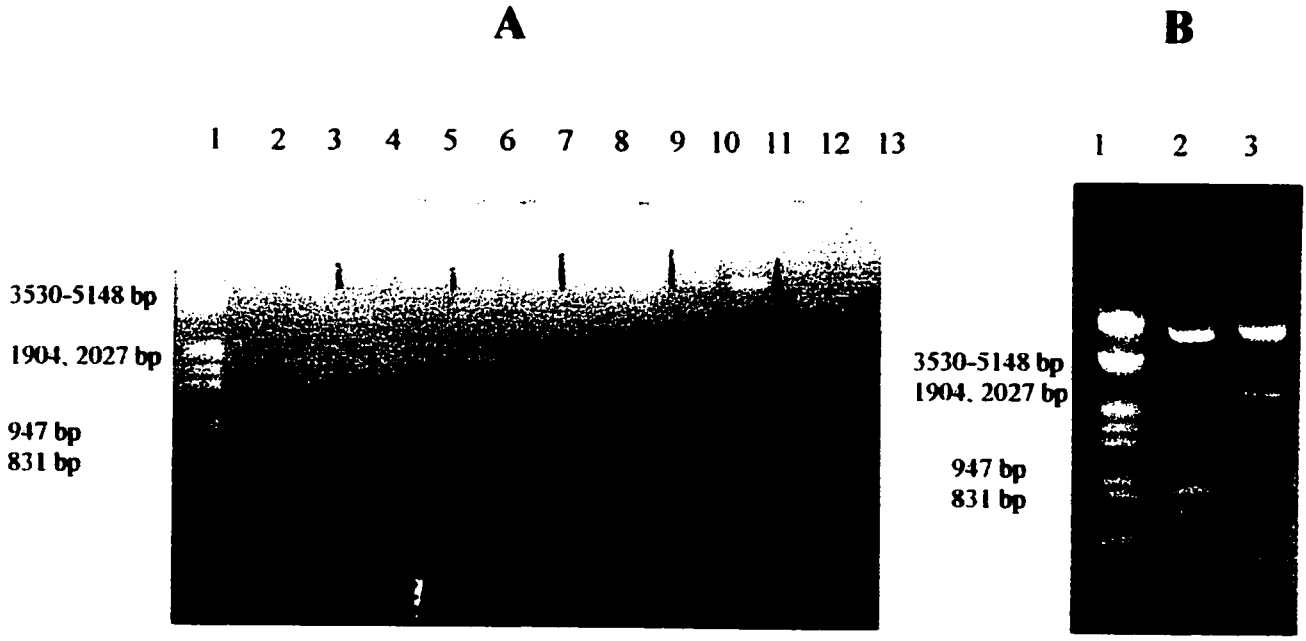



**Figure 23:** Agarose gel representing the restriction digestion of the expression vector pBIN35S35SEmGFP containing the *Arabidopsis* tRNA nucleotidyltransferase cDNAs with *Sac*I.

(A) Agarose gel representing the restriction digestion of the expression vector pBIN35S35SEmGFP-ARACCA-Long with *Sac*I

Lane 1: *Eco*RI-*Hind*III Lambda marker
Lane 2: Uncut pBIN35S35SEmGFP (control)
Lane 3: Digested pBIN35S35SEmGFP (control)
Lane 4,6,8,10 and 12: Uncut plasmid DNA
Lane 5,7,9 and 13: Digested plasmid DNA resulting in no insert
Lane 11: pBIN35S35SEmGFP containing the *Arabidopsis* cDNA starting from ATG1.

(B) Agarose gel representing the restriction digestion of the expression vector pBIN35S35SEmGFP-ARACCA-Short with *Sac*I

Lane 1: *Eco*RI-*Hind*III Lambda marker
Lane 2: Digested pBIN35S35SEmGFP containing the insert in the wrong orientation.
Lane 3: Digested pBIN35S35SEmGFP containing the *Arabidopsis* tRNA nucleotidyltransferase cDNA starting from ATG3 in the correct orientation.

DMIRBE confocal microscope. The protoplasts and protoplasts containing the plasmid encoding GFP alone were used as controls. Protoplasts lacking GFP showed little fluorescence when excited with light at 488 nm (Figure 24, panel A) while the cells carrying GFP fluoresced with GFP being dispersed throughout the cytoplasm and nucleus (Figure 24, panel B). When cells carrying pBIN35S35SEmGFP-ARACCA-Short (*Arabidopsis* tRNA nucleotidyltransferase lacking the targeting signal) were viewed, fluorescence was found throughout the cytoplasm and nucleus (Figure 24, panel C). On the other hand, pBIN35S35SEmGFP-ARACCA-Long (*Arabidopsis* tRNA nucleotidyltransferase containing the targeting signal) is clearly not found throughout the cytoplasm but in particular locations in the cell, possibly the mitochondria or chloroplasts (Figure 24, panel D). Autofluorescence makes it difficult to make any comments about the presence of GFP in the chloroplast (Figure 24, panel E-H).

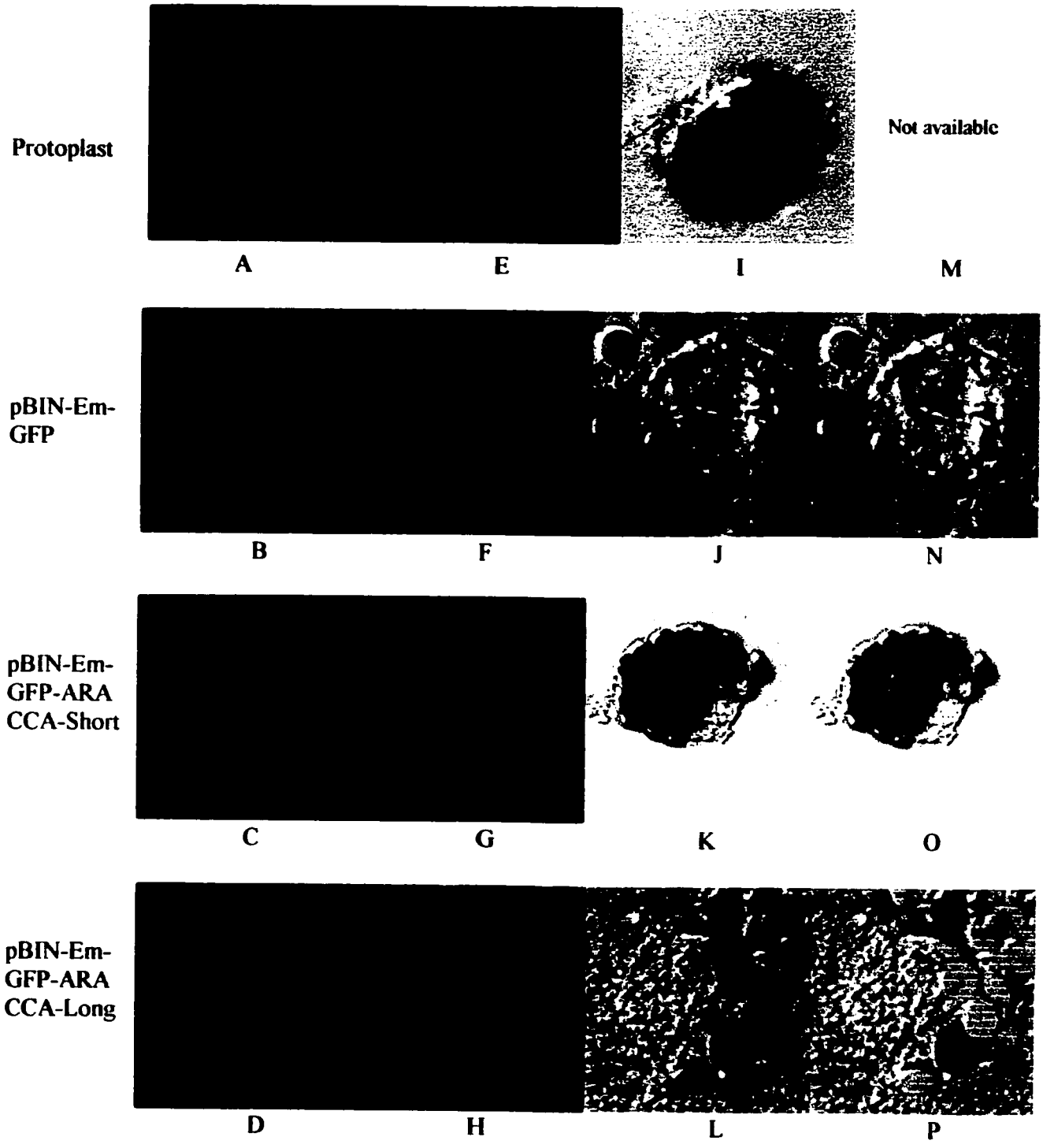



**Figure 24:** Confocal microscopy images of pBIN-EmGFP transformed into tobacco protoplasts. Samples are excited at 488 nm. Images (A-D) represent pictures taken with the green filter (capture wavelength 500-530 nm), (E-H) represent pictures taken with the red filter (capture wavelength 670-700), (I-L) represent pictures taken under phase contrast and (M-P) represent the overlay of the three images.

## Discussion

A cDNA coding for the *Arabidopsis* homologue of the yeast *GIM1* gene product was isolated using the yeast two-hybrid system (Gu, 2000). The predicted function of the *Arabidopsis GIM1* gene product is currently unknown although the significant similarity with both the yeast and mouse Gim1p's (Figure 6) may suggest a similar function for this protein in all of these organisms. A comparison of the *Arabidopsis GIM1* cDNA with the *Arabidopsis GIM1* gene suggests that the entire open reading frame coding for Gim1p has been isolated. In addition, the size of the predicted *Arabidopsis* Gim1p is in good agreement with the predicted sizes of Gim1p in yeast and mouse. Previous studies have shown that in other organisms (Geissler *et al.*, 1998), Gim1p is part of a larger complex that functions in the formation of the cytoskeleton (specifically in the formation of tubulin, the subunits of microtubules). If this protein plays a similar function in *Arabidopsis* it seems logical that this protein would be present in all tissues. To determine if the *Arabidopsis GIM1* gene was expressed in many tissues in *Arabidopsis*, cDNA was isolated from cauline leaves, rosette leaves, floral leaves and flowers and PCR amplified resulting in a fragment of expected size for the mRNA (compare to the cloned cDNA control in pJS19) (Lane 5, Figure 19B). As expected, the *GIM1* gene is being transcribed in all tissues. That these PCR products reflect the mRNA and not the gene itself is evident because the presence of an intron in the gene sequence results in a 700 bp product being produced when genomic DNA is used as a template for PCR (Figure 19A).

## 1. Structural characterization of the *Arabidopsis* Gim1p

In yeast, the family of GIM proteins shows a higher degree of similarity to their counterparts in other organisms than to each other, suggesting that if these proteins do have different functions then these different functions might be conserved within these organisms. Furthermore, the high degree of sequence conservation between the *GIM1* gene products in eukaryotic organisms (Figure 6) and the conservation of the protein with its archaea homologue suggest a conserved function for this protein.

In order to determine whether or not the conservation at the level of primary sequence was reflected in conservation of higher order structure, the *Arabidopsis* Gim1p was analysed by circular dichroism (CD) spectroscopy, an analytical technique that can elucidate secondary structural properties of a particular protein of interest by measuring the difference in absorption between left and right handed circularly polarized light (Alder *et al.*, 1973). Proteins and nucleic acids contain elements of asymmetry and thus exhibit distinct CD signals. A protein CD signal arises from peptide bonds, histidine, cysteine, tryptophan, tyrosine and phenylalanine residues (Greenfield and Fasman, 1996). In this work, we used Far UV CD (190 nm and 260 nm) and structure prediction programs to determine secondary structural characteristics of the *Arabidopsis* Gim1p to compare it to the published archael model (MtGimβ) (Leroux *et al.*, 1999). The *Arabidopsis* Gim1p is a relatively small protein of only 129 amino acids and its similarity in size and primary sequence with its *M. thermoautotrophicum* (121 amino acids), mouse (127 amino acids) and yeast (114 amino acids) homologues suggests a similar secondary structure for all of these proteins. The scans acquired for the *Arabidopsis* Gim1p (Figure

9) were compared to those published for the MtGimβ protein. The *Arabidopsis* Gim1p spectrum at 25°C has clear troughs at 222 nm and 209 nm, typical of proteins with high α-helical content and consistent with what was observed for the MtGimβ protein (Leroux et al, 1999). Using prediction programs such as Peptool lite (www.uib.no/People/mblpp/links_old/software.htm) we predicted that the *Arabidopsis* Gim1p would be 86% α-helical, in good agreement with data presented for the archaeal Gimβp. From figure 9 we also see that as the temperature is gradually increased (25°C to 90°C) there is a decrease in trough intensity at 222 nm and 209 nm indicating that the protein is being denatured. By plotting the relative molar ellipticity intensity at 222 nm versus temperature, we can monitor unfolding patterns and determine the melting temperature ($T_m$) when 50% of the protein is unfolded (Figure 10). The plot clearly demonstrates that the *Arabidopsis* Gim1p unfolds gradually in a one state process with no distinct intermediate. Based on these data the melting temperature was calculated as 42°C. This $T_m$ is considerably lower than the $T_m$ (> 60°C) published by Siegert *et al.* (2000) for the MtGimβ. This is perhaps not surprising as *M. thermoautotrophicum* is a thermophilic bacterium. However, this also is a low melting temperature as compared to other proteins of similar size, *e.g.*, RNase T1 (114 amino acids, $T_m$ =48°C) (Myers *et al.*, 1997) or the regulatory subunits of aspartate transcarbomylase (154 amino acids, $T_m$ =50°C) (Peterson *et al.*, 1994). The low $T_m$ for the *Arabidopsis* Gim1p may suggest that this protein is unstable when not associated with the GIMC. A difference in $T_m$'s for a protein either free or in a complex also has been seen with the catalytic and regulatory subunits of aspartate transcarbomylase (ATCase) (Peterson and Schachman, 1991, Peterson *et al.*, 1994). The isolated regulatory and catalytic subunits unfold at

temperatures of 50°C and 60°C, respectively. However, when present as part of the holoenzyme complex their $T_m$'s increase by 10°C and 5°C, respectively (Peterson and Schachman, 1991, Peterson *et al.*, 1994). To better clarify the stability of the *Arabidopsis* Gim1p as part of GIMC it would be of interest to determine the $T_m$ value for Gim1p as part of the complete *Arabidopsis* GIM complex.

One *caveat* that must remain in the analysis of these data is the fact that the *Arabidopsis* Gim1p generated here has an additional 36 amino-terminal amino acids including 6 histidine residues that result from expressing this protein in the heterologous expression system. It is possible that the additional amino acids alter the CD spectrum or the melting temperature and so these experiments need to be repeated with the protein lacking these amino acids. In any event, these data suggest that the *Arabidopsis* Gim1p homologue is similar to Gim1p from other organisms, not only at the amino acid level but also structurally. Based on this it seemed reasonable to suggest that it might also have a common function in these organisms.

**2. Complementation of the yeast *GIM1* defect with the *Arabidopsis* Gim1p**

A key feature of genes involved in microtubule biogenesis is that mutations in them result in cold and benomyl supersensitive phenotypes (Stearns *et al.*, 1990, Chen *et al.*, 1994, Archer *et al.*, 1995, Tian *et al.*, 1997). This is the case with many of the GIM deletions in yeast (Geissler *et al.*, 1998). The strong similarity of the *GIM1* genes among different species suggests that a defect in one *GIM1* gene could be complemented by its homologue from another organism. Geissler *et al.* (1998) clearly demonstrate this by

using the murine and human *GIM1* homologues to eliminate the cold and benomyl supersensitivity present in a yeast strain bearing a disrupted *GIM1* gene. They observed in both serial dilution growth on plates and in growth curves, that the yeast Δ gim1::kanMX4 mutation can be complemented by either its mouse or human homologue. Moreover, they showed that a defect in any of the GIM family of genes results in a reduced growth rate as compared to the wild-type at low temperature. We wanted to define the function of the *Arabidopsis GIM1* gene and initially attempted to show that it would complement the same *GIM1* defect Geissler *et al.* (1998) had generated. To do this a cDNA coding for the *Arabidopsis* Gim1p was cloned into the vector p415-ADH and transformed into the yeast strain SGY101 lacking a functional Gim1p. At the same time we also transformed with p415-ADH (no insert) alone (negative control) and pSG55 and pSG72 (positive controls). In contrast to what had been shown by Geissler *et al.* (1998), when the yeast strains were patched on YPD medium (30°C), we found that there was no difference between strains lacking the *GIM1* gene (p415-ADH) or carrying *GIM1* genes from *Arabidopsis*, mouse or yeast (Figure 13A). This is a clear indication that the *GIM1* gene is not essential at this temperature. The yeast strains were also incubated at reduced temperatures (14°C and 23°C) because microtubule biogenesis is affected as the temperature is decreased (Chen *et al.*, 1994). As expected, all of the yeast strains grew more slowly at the lower temperatures but no significant differences were seen in growth rates among any of the strains. The negative control (no insert [p415-ADH]) had fewer cells at 14°C than all the other strains but the differences were negligible (Figure 13A). This is also different from the data presented by Geissler *et al.* (1998) who showed that there was no growth of the negative control at 14°C. These differences between what I

observed and what Geissler *et al.* (1998) reported may result from the way that the data were collected. I used replica plating to transfer my cells while Geissler *et al.* (1998) performed serial dilutions and thus fewer cells were transferred to the plates. The question of the effect that cell number transferred to each plate might have on the interpretation of the data will be addressed later.

To enhance the cold sensitive effect, the yeast strains were plated on YPD containing 2.5 μg/ml of benomyl which has been shown to amplify the cold sensitivity. Under these conditions, Geissler *et al.* (1998) reported that growth of the strain bearing a disrupted *GIM1* gene (no insert [p415-ADH]) alone was effected at 14°C, 23°C and 30°C. Again, we report that there is no growth defect at 30°C, but the data obtained at 14°C and 23°C (Figure 13B) are consistent with what has been reported (Geissler *et al.*, 1998). As expected, the growth of the positive control strains carrying the yeast (pSG55) and mouse (pSG72) *GIM1* sequences was not affected. In addition, the strains carrying the *Arabidopsis GIM1* homologue (pJS19 and pJS19H) grew at a rate equal to that of the positive controls indicating that the *Arabidopsis GIM1* gene product is able to complement the yeast defect. These data indicate that the *Arabidopsis GIM1* gene product can replace the yeast copy of this protein. This observation, taken with evidence identifying *Arabidopsis* proteins corresponding to each of the yeast GIMC subunits except Gim6p which seems to be present only in yeast (Table 1), suggests that like in other organisms this complex also exists in *Arabidopsis* where it may have a similar function.

### 3. Interaction of *Arabidopsis* Gim1p with the *Arabidopsis* tRNA nucleotidyltransferase

The isolated GIMC has been extensively studied in yeast (Geissler *et al.*, 1998). Genetic interaction studies (Geissler *et al.*, 1998) have demonstrated that Gim1p interacts with two distinct classes of gene products, one which is involved in protein folding in general and the other which plays a role more specifically in microtubule biogenesis. Interestingly, the *GIM1* gene product is not essential in yeast (Geissler *et al.*, 1998) suggesting that under certain conditions there are other proteins with shared function. Double and triple knockout experiments with subunits of GIMC (*e.g.*, Δgim1 with any one of Δgim2/pac10, Δgim3, Δgim4 or Δgim5 and Δgim1/2/3) have demonstrated that the strain is still viable and shows a cold sensitive phenotype (Geissler *et al.*, 1998) suggesting that knocking out one of the GIM genes is as effective as knocking out more than one in causing a complete loss of function, at least in actin and tubulin folding (Siegers *et al.*, 1999, Llorca *et al*, 2001). These data imply that the Gim proteins act in unison as a complex to perform their various roles in microtubule biogenesis. Even if this is the case we cannot rule out possible functions for the components of this complex in addition to microtubule formation (Leroux *et al.*, 1999).

The yeast two-hybrid system (Fields and Song, 1989) is used to identify *in vivo*, genes encoding proteins that physically interact. This *in vivo* system monitors the ability of a protein attached to the DNA binding domain of *GAL4* to interact with another protein attached to the activation domain of *GAL4* reconstituting *GAL4* and activating expression of a reporter gene (*e.g.*, *HIS3*). It is evident from our preliminary studies that

the *Arabidopsis* Gim1p is structurally similar to its yeast and mammalian homologues and, therefore, will likely have a similar function. As the best characterized function of this protein seems to be in microtubule formation it was surprising that a two-hybrid screen with tRNA nucleotidyltransferase identified Gim1p. There is no obvious connection between tRNA nucleotidyltransferase and microtubules. However, given the more general role of Gim1p as a molecular chaperone in yeast (Geissler *et al.*, 1998), it is perhaps less surprising that its *Arabidopsis* homologue can interact with tRNA nucleotidyltransferase possibly as a molecular chaperone. There is clearly a precedent for molecular chaperones, *e.g.*, HSP70, COM70 and MSF being involved in protein folding, assembly and intracellular trafficking (Hachiya *et al.*, 1993, 1994). If tRNA nucleotidyltransferase is directed to specific locations within the cell Gim1p may, like these proteins, play a role in this process. The *Arabidopsis* Gim1p interacts with the *Arabidopsis* tRNA nucleotidyltransferase containing the N-terminal potential mitochondrial or chloroplast targeting signal and not with tRNA nucleotidyltransferase lacking this sequence (Gu, 2000). In our lab we have shown a reduced growth rate for yeast cells overexpressing either the lupin or *Arabidopsis* tRNA nucleotidyltransferase containing this additional N-terminal targeting information (Joyce, unpublished). Mitochondrial and chloroplast targeting signals have been hypothesized to reduce the folding efficiency (see review Luzikov, 1999) or how tightly a protein can fold (Pugsley, 1989) to allow it to remain in a loose conformation so that it can more easily be transported across the chloroplast or mitochondria membranes (Bandlow *et al.*, 1998). Perhaps the long forms of the plant tRNA nucleotidyltransferase interact with Gim1p in yeast simply because they are improperly or incompletely folded and Gim1p plays some

role in their folding. In yeast Gim1p carries out its function as part of a larger complex (GIMC) (Geissler *et al.*, 1998) and this complex has been implicated in protein folding (Leroux and Hartl, 2000). As mentioned previously, the *M. thermoautotrophicum* homologue of GIMC has been shown to interact with a number of unfolded proteins (Leroux *et al.*, 1999, Leroux and Hartl, 2000). We wanted to know if GIMC was required in yeast for the interaction of the *Arabidopsis* Gim1p with tRNA nucleotidyltransferase or whether Gim1p played a role in tRNA nucleotidyltransferase synthesis, assembly, folding, transport or targeting that was not dependent on GIMC. To address this question we disrupted a gene, *GIM5*, coding for one of the other components of GIMC. Gim5p is part of the alpha subunit of the complex (Figure 5), therefore, removing this should make it impossible for the complex to assemble. As expected, the *GIM5* knockout strain was cold and benomyl sensitive (data not shown). This indicates, as had been proposed previously by Geissler *et al.* (1998), that a *GIM5* knockout like a *GIM1* knockout has a defect in microtubule assembly indicating a non-functional GIMC. When either HF7c or HF7c GIM5::TRP1 was transformed with pAS2-ARACCA-Long and pACT108A no two-hybrid interaction was observed. Previous experiments (Gu, 2000) had identified p108A through two-hybrid interaction with another protein but not with *Arabidopsis* tRNA nucleotidyltransferase. This lack of interaction in HF7c GIM5::TRP1 indicates that disrupting *GIM5* did not generate a false positive two-hybrid result. When both the pAS2-ARACCA-Long and pACT14A or pACT19A (Gim1p) were transformed into the yeast strain HF7c the expected interaction was observed (Gu, 2000) (Table 7). When these same plasmids were transformed into HF7c GIM5::TRP1 the results indicated that the product of pACT14A still interacted with tRNA

nucleotidyltransferase confirming that the two-hybrid interaction, in general terms, is not affected by the disruption of *GIM5*. These data also indicate that although the *GIM5* gene was disrupted with *TRP1* (and, therefore, there is now a functional chromosomal copy of *TRP1*), the plasmid pAS2-ARACCA-Long which also carries the *TRP1* gene as a selectable marker is maintained if in combination with the second two-hybrid plasmid it helps to confer histidine prototrophy. To this point the results were as expected. The most interesting results were generated when pAS2-ARACCA-Long and pACT19A (Gim1p) were transformed in HF7c and HF7c GIM5::TRP1. As expected histidine prototrophy was seen in HF7c indicating that the two proteins interacted. In contrast, no interaction was seen in HF7c GIM5::TRP1. This suggests that the *Arabidopsis* Gim1p does not function alone in its interaction with tRNA nucleotidyltransferase, but requires at least Gim5p and probably the whole GIMC. Although based on our data with pAS2-ARACCA-Long and pACT19A in HF7c GIM5::TRP1, it seems unlikely that the lack of a positive two-hybrid result with pAS2-ARACCA-Long and pACT19A (Gim1p) in HF7c GIM5::TRP1 is simply the result of the loss of pAS2-ARACCA-Long (since it is no longer required for growth on trp$^-$ medium) or the inability to transform this plasmid into the strain, we can not as yet formally exclude this possibility. To address this concern I am using two approaches: 1) I am disrupting *GIM5* with *Candida glabrata ADE2* and 2) I am generating a new two-hybrid pAS2 derivative which contains *ADE2* as a selectable marker instead of *TRP1* (Hanic-Joyce, 1998). With either of these two approaches I will be able to repeat this experiment eliminating the concern of having the same marker in the chromosome and the plasmid. Also, I would like to disrupt *GIM1* in HF7c. This would allow two questions to be addressed: 1) Can the Gal4p-Gim1p fusion protein

complement the cold-sensitive defect and 2) will the *Arabidopsis* Gim1p interact with tRNA nucleotidyltransferase under these conditions?

The role of the interaction of Gim1p and tRNA nucleotidyltransferase in tRNA maturation, synthesis or targeting remains to be elucidated. It is interesting that for the two-hybrid interaction to occur the two interacting proteins must be in the yeast nucleus (to induce gene expression) yet GIMC has been reported in the cytosol (Geissler *et al.*, 1998). The fact that Gim1p has a leucine zipper characteristic of DNA binding proteins (Shang *et al.*, 1994) may suggest that this protein also has a function in the nucleus. The fact that this two-hybrid interaction is only seen when Gim5p is present may indicate that this function is as part of GIMC.

In yeast, Gim1p and even GIMC may be involved in correcting the misfolding of tRNA nucleotidyltransferase. Perhaps the incomplete or incorrect folding of the *Arabidopsis* tRNA nucleotidyltransferase containing its targeting signal allows it to remain associated with Gim1p long enough for a two-hybrid interaction to be registered in yeast. One might also suggest that if Gim1p is associated with GIMC and GIMC is required for other cellular functions (*e.g.*, tubulin assembly) then having GIMC filled with *Arabidopsis* tRNA nucleotidyltransferase (when overexpressed) would not allow this complex to interact with other proteins and could lead to the reduced growth rate that we have observed. To further address the role of GIMC in tRNA nucleotidyltransferase synthesis, targeting or function it would be useful to see if the yeast tRNA nucleotidyltransferase interacts with the yeast Gim1p. If the yeast tRNA nucleotidyltransferase was shown to interact with the yeast Gim1p then this would suggest a role for this interaction that was conserved between organisms. In contrast, if

no interaction was observed between these two proteins in yeast this might suggest either that the interaction that we observed between the *Arabidopsis* proteins was an artifact of producing these proteins in yeast or that the interaction is specific for the *Arabidopsis* proteins. To further explore these possibilities it would be interesting to mix and match these proteins, for example, to see if *Arabidopsis* tRNA nucleotidyltransferase interacts with the yeast Gim1p or *vice versa*. Data from Uetz *et al.* (2000a, 2000b) and Ito *et al.* (2001) have not identified an interaction between yeast Gim1p and yeast tRNA nucleotidyltransferase. The significance of this observation remains to be elucidated. More research, particularly, the analysis of protein-protein interaction in *Arabidopsis* will be required to determine what role, if any, Gim1p plays in *Arabidopsis* in the synthesis, folding, targeting, transport or function of tRNA nucleotidyltransferase.

**4. Determination of relative growth rates from stationary phase**

Our replica plating results indicated that the *Arabidopsis GIM1* homologue could complement the yeast *gim1* defect. To more precisely define how efficiently the *Arabidopsis GIM1* homologue could function in yeast, growth curves comparing the growth rates of yeast cells producing the yeast wild-type or *Arabidopsis* Gim1p were generated. This experiment may also help to address the differences in phenotypes we saw compared to Geissler *et al.* (1998). If the observed differences resulted from transferring too many cells during replica plating as compared to using serial dilutions as Geissler *et al.* (1998) did then this procedure may allow me to better compare my results to those of Geissler *et al.* (1998). Because benomyl amplified any effects seen, growth

curves were generated only for the cells grown in YPD medium plus benomyl. I proceeded to grow each yeast strain in YPD containing benomyl to determine relative growth rates. All strains are compared to the yeast strain carrying its own *GIM1* gene on p415-ADH. Growing the cells at 30°C resulted in a similar growth rate for each of the homologues and even the strain bearing the disrupted *GIM1* gene grew fairly well (Figure 14). By comparing the slopes of the growth curves, the disrupted strain grows approximately 84% as well as the strain carrying the yeast *GIM1* gene once again indicating that this gene is not essential at the permissive temperature (Figure 25). Based on these data I now can confirm, as Geissler *et al.* (1998) reported, that at 30°C there is a change in growth rate between the *GIM1* disrupted strain and the strain carrying the yeast *GIM1* gene (pSG55) although both strains do grow at this temperature. When the temperature was decreased to 23°C, there was a decrease in growth rate of the strain bearing the disrupted *GIM1* gene as compared to the wild-type gene (Figure 15) Interestingly, not only is doubling time affected but also the level of saturation of the stationary phase culture. A possible explanation of this observation is that although most cells are still dividing, more die as the experiment progresses as compared to the control. When the same experiment was conducted at 14°C the strain lacking the *GIM1* gene exits lag phase very slowly while the strain with the yeast *GIM1* gene, although growing slower than at 23°C and 30°C, grows as expected (Figure 16). In all of these examples the mouse *GIM1* (when tested) was able to restore the growth to approximately wild-type levels. Similarly, as expected, the *Arabidopsis GIM1* did so as well.

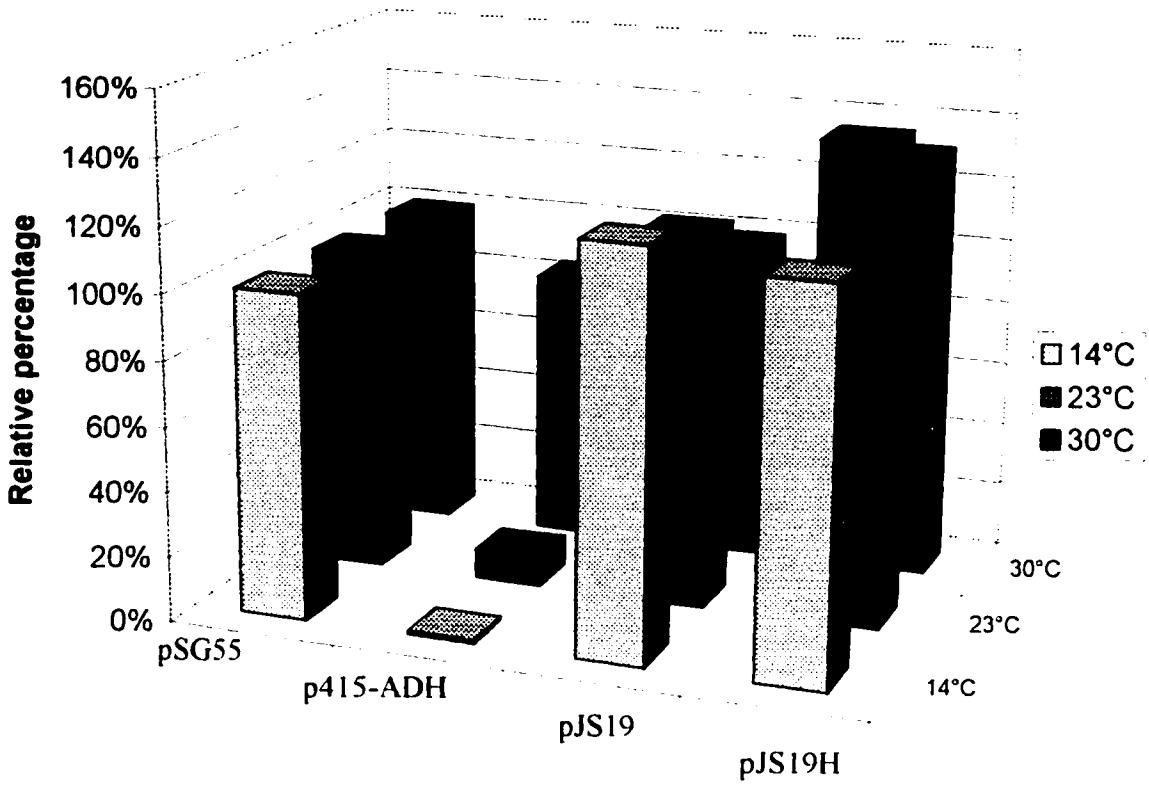



**Figure 25:** Bar graph representing the relative growth rates of the yeast strains containing the indicated plasmids. The values of the strain carrying the yeast *GIM1* gene have been normalized to 100% and all values adjusted accordingly. The plasmids are: p415-ADH (no insert), pSG55 (yeast *GIM1*), pJS19 (*Arabidopsis GIM1*), pJS19H (*Arabidopsis GIM1* with 6X His tag).

The inability of the strain bearing the disrupted *GIM1* gene to exit from stationary phase was an interesting result that had not been reported before. This same phenotype has been reported previously. For example, complexes composed of multiple subunits have been identified (Schramm *et al.*, 2000, Drebot *et al.*, 1987), that when unable to come together prevent cells from exiting stationary phase. Clearly, the removal of a particular component from GIMC, Gim1p confers a similar phenotype.

An interesting observation is that at 30°C the yeast strain carrying the *Arabidopsis GIM1* (pJS19) grows at a rate that is the same as that of the strain carrying the yeast *GIM1* (pSG55) while the strain carrying the *Arabidopsis GIM1* with the additional 6X His tag (pJS19H) grows even more quickly (Figure 25). When the strains are compared at 23°C the strain carrying the *Arabidopsis GIM1* (pJS19) grows more quickly than the strain with the yeast GIM1 (pSG55) and the strain expressing the his-tagged *Arabidopsis* Gim1p (pJS19H) is still the fastest doubling (Figure 25). Finally, at 14°C both the strains carrying the *Arabidopsis GIM1* double faster than the yeast *GIM1* strain although no difference is seen between the two. Although the data sets are too small to attach any statistical significance to these observations it is interesting in that the yeast cells with the *Arabidopsis GIM1* gene containing the 6X His tag also exit lag phase before the yeast control at all temperatures. This could be due to the extra 6X His tag at the carboxy terminus adding 6 positively charged amino acids which may allow for better interaction with associated protein or which may increase the stability of the *Arabidopsis* Gim1p. It is unclear whether the extra amino acids confer a structural advantage to assist in protein-protein interactions or to prevent premature degradation (Varshavsky *et al.*, 2000).

**5. Determination of relative growth rate from exponential phase**

We found that a deletion of the *GIM1* gene seems to prevent the yeast strains from coming out of stationary phase ($G_o$). It is evident from Figure 16 that at 14°C a stationary phase culture of yeast cells lacking the *GIM1* gene shows only a small increase in absorbance over time suggesting a lack of cell division. To separate the inability of cells to exit $G_o$ from their ability to divide, the effects of cold and benomyl were tested on cells that had already entered exponential phase. This was done by adding benomyl and shifting the temperature during early exponential phase. The yeast strains carrying the *Arabidopsis*, yeast and mouse *GIM1* genes grew more slowly due to the temperature shift but continue to divide normally based on an increase in $OD_{640}$ (Figure 17). The strain lacking the *GIM1* gene also slows in growth rate compared to the yeast strains carrying the *GIM1* genes but its ability to continue to divide indicates that the effects of the cold and benomyl are not as dramatic on cells that are actually dividing as they are on cells in $G_o$. This was somewhat surprising due to the fact that microtubules disassemble in the presence of cold and benomyl when the *GIM1* gene is absent (Ursic and Culbertson, 1991 and Geissler *et al.*, 1998). In *S. cerevisiae* the series of events (START) required to initiate the cell cycle is regulated by checkpoints which control the ability of the cell to continue to divide (Dahmann *et al.*, 1995, Nasmyth, 1996). One such check point involves the kinetochore which mediates the attachment and movement of chromosomes along microtubules during cell division (Gruneberg *et al.*, 2000). When microtubules are not functional such as in a strain lacking the *GIM1* gene grown at 14°C, the kinetochore fails to attach to the microtubules thus arresting the cell at the mitotic checkpoint. A

possible explanation for the observation of continued cell division in the strain bearing the disrupted *GIM1* gene at 14°C is that the cells that are already in the process of dividing at the time of the block will continue to divide until they reach START while cells at START will be blocked. The apparent growth of the yeast strain bearing the disrupted *GIM1* gene may be due to the fact that the yeast cells accumulate at START but not necessarily at any other part of the cell cycle. One example, of this type of response is the *rpo21-4* mutation which is a temperature sensitive mutation for RNA polymerase II. It was found that this mutation does not impede cell cycles in progress, but rather blocks the START regulatory step to initiate a new cell cycle (Drebot *et al.*, 1993). This explains why the cells seem to continue to divide for an extended period of time. To address some of these questions it would be pertinent to check the morphology of the cells at the various temperatures and times to see if they are forming buds slowly or if they are not budding at all. It might also be of interest to block the cell cycle using chemicals (*e.g.*, A or α-factor prior to the temperature shift and addition of benomyl) to synchronize the cells and see at what stage they arrest in the cell cycle.

**6. Phenotypic analysis of transformed *Arabidopsis* plants**

The enzyme, tRNA nucleotidyltransferase, is required for the addition of AMP and CMP residues onto immature tRNAs that have an incomplete CCA sequence. The *S. cerevisiae*, tRNA nucleotidyltransferase is produced from a single gene and has been shown to function in multiple cellular locations (Chen *et al.*, 1992). Preliminary experiments indicate that this may also be the case for other organisms including plants.

There is currently very little information on how this enzyme is targeted to various compartments. Proteins that are shared between different cellular compartments often result from different transcription initiation sites being used such that multiple in frame start codons are generated in mRNAs from a single gene (Small *et al.*, 1998). To localize the *Arabidopsis* tRNA nucleotidyltransferase in the different subcellular compartments, various plasmid constructs: 1) the entire *Arabidopsis* tRNA nucleotidyltransferase coding region including the potential organellar targeting signal fused to GFP, 2) the *Arabidopsis* tRNA nucleotidyltransferase coding region lacking the potential organellar targeting signal fused to GFP and 3) only the potential organellar targeting signal (bold in Figure 2) fused to GFP, were generated in pBIN35SmGFP4 and transformed into *Arabidopsis* (Figure 8). *Agrobacterium* mediated transformation was used so that the DNA of interest was transferred at random into the plant nuclear genome. On average 10 000 - 15 000 seeds per plant construct were screened for insertion events. After the first round of selection (F1) <1% of the seeds had at least one insertion event *i.e.* were able to grow in the presence of kanamycin. It is difficult to determine how many insertion events occurred in each plant. After the second round of selection (F2) there was great variation between each line (Table 8). The ratio of transformed to untransformed seeds varied from 50% in the plants transformed with only the *Arabidopsis* organellar targeting signal to 100% in plants transformed with the *Arabidopsis* cDNA containing the targeting signal. At the F3 generation there was no variation in each construct and all the seeds were green (Table 8). This was surprising because at this point with insertion at a single locus one would expect to see a Mendelian genetic result where 25% of the progeny would be sensitive to the antibiotic. These results suggest that in all of the lines selected

there were multiple insertion events. Overall, the plants were monitored on a weekly basis and demonstrated no phenotypic abnormalities during growth. Unfortunately, using these plasmids derived from pBIN35mGFP4 resulted in a very faint fluorescent signal from GFP which was difficult to view. To overcome this deficiency I began building new constructs with a stronger promoter and brighter fluorescing GFP.

### 7. Localization of Arabidopsis tRNA nucleotidyltransferase in tobacco protoplasts

As discussed above, the localization of *Arabidopsis* tRNA nucleotidyltransferase to various cellular compartments is of great interest to us. To address this question I used plasmids derived from pBIN35S35SEmGFP and a transient transformation system utilizing tobacco protoplasts. As with the stably transformed lines I used plasmids carrying the long form of tRNA nucleotidyltransferase (containing the amino terminal targeting signal), the short form of tRNA nucleotidyltransferase (lacking this information). Initially I was able to transiently transform tobacco protoplasts with CCA-long and CCA-short and I am generating a plasmid carrying the targeting signal alone. As expected before transformation, the protoplasts showed limited green fluorescence (Figure 24, panel A) but did show autofluorescence from chlorophyll (Figure 24, panel E) as expected. Comparing these results to the protoplasts that contained pBIN35S35SEmGFP (Figure 24, panel B) it appears that in these plants GFP is distributed throughout the nucleus and the cytosol. While the location of the nucleus can be determined from the phase contrast photos (Figure 24, panel J) it will be important to confirm its location with nucleus-specific markers. As GFP is not targeted to any

particular region of the cell it is not surprising that it would be found in the cytosol and the nucleus. GFP with a molecular mass of 25 000 Da is small enough to enter the nucleus through the nuclear pore complex even though it lacks an NLS, but it should not enter mitochondria or chloroplasts or the vacuole as it lacks targeting information to direct it to these intracellular destinations. If one compares panels B and J, Figure 24, it is clear the there is no green fluorescence associated with the vacuole. The presence or absence of green fluorescence in the chloroplast is slightly more problematic (Figure 24, compare panels B and J). A Z scan through the cell (data not shown) makes it more apparent that there is no green fluorescence in the chloroplast. The small size of mitochondria makes it difficult at this point to determine whether or not there is any GFP in these organelles. Cells carrying pBIN35S35SEmGFP-ARACCA-Short (*Arabidopsis* tRNA nucleotidyltransferase lacking the targeting signal) showed similar results to EmGFP alone with green fluorescence found primarily in the cytosol and nucleus (Figure 24, panel C) and none in the chloroplast or vacuole (Figure 24, panel O). The large size of this fusion protein means that it must contain a nuclear localization signal to be transported into the nucleus. This is the first evidence that tRNA nucleotidyltransferase may be present in the nucleus in plants as it is in yeast. Finally, cells carrying pBIN35S35SEm-ARACCA-Long (*Arabidopsis* tRNA nucleotidyltransferase containing the targeting signal) clearly show that green fluorescence is primarily not distributed throughout the cytosol but rather located in distinct aggregations in the cytosol and areas surrounding the chloroplast (Panel D). The punctate staining is characteristic of mitochondrial localization (Kohler *et al.*, 1997) as is the location of these organelles in the cell. Without a second mitochondrial-specific marker the identity of these fluorescent

aggregations can not be confirmed, but their presence in the protoplasts carrying the gene coding for the potential targeting signal and their absence from all of the other protoplasts again supports their identity as mitochondria. Again, the first experiment to further confirm this localization is to carry out this experiment in plants with a known mitochondrial marker. Also, I will complete construction of the plasmid carrying the targeting signal from the *Arabidopsis* tRNA nucleotidyltransferase fused to GFP and transform that into plants. These preliminary experiments suggest that the *Arabidopsis* tRNA nucleotidyltransferase is found in the nucleus, cytosol and mitochondrion. There are other examples of plant enzymes shared among these locations, *e.g*, glutathione reductase (Creissen *et al.*, 1995), carrot dihydrofolate reductase-thymidylate synthase and valyl-tRNA, threonyl-tRNA, alanyl-tRNA and glycyl-tRNA synthetases (see Small *et al.*, 1998 for review) and they are expressed in a similar manner to tRNA nucleotidyltransferase, *i.e.*, by producing two types of transcripts, one which can code for the mitochondrial targeting signal and one which cannot. If we assume that as these preliminary results indicate this tRNA nucleotidyltransferase is not found in chloroplasts then that raises the intriguing question of where is the gene coding for the chloroplast enzyme? There is only one gene in the *Arabidopsis* genome that shows significant similarity with tRNA nucleotidyltransferases from other organisms (Theologis *et al.*, 2000). Further experiments will be required to rule out that this protein is found in the chloroplast as well. Autofluorescence makes it difficult to view the presence of green fluorescence in the chloroplast. It is particularly difficult to precisely define the location of a protein that must exist in some form or other in the cytosol, nucleus, mitochondrion and perhaps even chloroplast.

## 8. Conclusion

In this work, an Arabidopsis homologue of the yeast *GIM1* gene has been further characterized and shows significant similarity to the yeast and mouse *GIM1* gene. The highly conserved homology between the *GIM1* family may indicate a similar fundamental role. Our findings demonstrate that the *Arabidopsis* Gim1p can complement a yeast Gim1p deletion. The fact that the *Arabidopsis* Gim1p can substitute for the yeast Gim1p in yeast suggests that these proteins share a common function.

In conjunction with GIMC, the *Arabidopsis* Gim1p is involved in the interaction with a cDNA encoding an *Arabidopsis* tRNA nucleotidyltransferase. This interaction between the *Arabidopsis* tRNA nucleotidyltransferase containing the amino terminal targeting signal with Gim1p along with the lack of interaction between the yeast tRNA nucleotidyltransferase and the yeast Gim1p suggests that this function may be unique to plants. Thus suggesting a possible role of GIMC in the intracellular transport and targeting of tRNA nucleotidyltransferase in *Arabidopsis*. To support my two-hybrid result I would like to demonstrate, using biochemical techniques such as CD and fluorescence, that there is an interaction between the *Arabidopsis* GIM complex and the *Arabidopsis* tRNA but not between the *Arabidopsis* Gim1p and tRNA nucleotidyltransferase.

We also have demonstrated that the *Arabidopsis* tRNA nucleotidyltransferase without the targeting signal is found in the nucleus and cytosol while the protein containing the amino terminal targeting signal is directed to more localized regions,

possibly the mitochondria although the chloroplast cannot be ruled out at this point. If it is only targeted to mitochondria then there may be an additional gene coding for tRNA nucleotidyltransferase for the chloroplast. It will be necessary in the future to clarify where it is targeted and possibly find the additional protein that is targeted to the chloroplast.

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