Confirmation of protein interactions involving tRNA nucleotidyltransferase from yeast two-hybrid data

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Abstract

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A transfer RNA (tRNA) requires the nucleotide sequence cytidine, cytidine, adenosine at its 3' terminus to be functional. These nucleotides are added by the enzyme ATP (CTP): tRNA-specific tRNA nucleotidyltransferase (tRNA nucleotidyltransferase). In eukaryotes, this enzyme which is made in the cytosol must be targeted to specific subcellular destinations for tRNA maturation. Directing proteins to defined intracellular locations requires targeting signals on the protein of interest as well as other helper proteins. Previous studies have shown that AraYml079wp and AraGim1p, the products of the *Arabidopsis* Atlg19130 and Atlg29990 genes, respectively, interact with plant tRNA nucleotidyltransferases containing potential mitochondrial or chloroplast targeting signals. In this study, a heterologous *E. coli* expression system was developed to produce tRNA nucleotidyltransferase and the interacting proteins. Using these proteins a number of *in vitro* techniques including far Western blotting, co-elution and cross-linking were used to confirm the yeast two-hybrid data. *In vitro* results using co-elution and far Western blotting supported the *in vivo* results for an interaction between lupin tRNA nucleotidyltransferase and AraYml079wp. A number of

experiments also were performed using a yeast *YML079w* deletion strain to try to elucidate a function for Yml079wp.

Acknowledgment

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Abbreviations

Ara1 or A1	*Arabidopsis* tRNA nucleotidyltransferase gene product starting from the first ATG
Ara3 or A3	*Arabidopsis* tRNA nucleotidyltransferase gene product starting from the third ATG
AraGim1p	*Arabidopsis* homologue of the yeast *GIM1* gene product
AraYml079wp	*Arabidopsis* homologue of the yeast *YML079w* gene product
bp	base pair
CCA	cytidine, cytidine, adenosine
DB domain	DNA binding domain
EDC	(1-ethyl-3-[3-dimethylaminopropyl]carbodiimide)
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
GIM	Gene Involved in Microtubule biogenesis
GST	Glutathione S-transferase
His	Histidine
KDa	Kilo dalton
Lup1 or L1	Lupin tRNA nucleotidyltransferase gene product starting from the first ATG
Lup2 or L2	Lupin tRNA nucleotidyltransferase gene product starting from the second ATG
NHS	N-succinimidyl ester
OD	Optical density
PAGE	PolyAcrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Rpm	Revolution per minute
SDS	Sodium Dodecyl Sulfate
TA domain	Transcription Activation domain
tRNA	transfer Ribonucleic Acids
tRNA nucleotidyltransferase	ATP(CTP):tRNA-specific tRNA nucleotidyltransferase
UAS	Upstream Activation Sequence
W	Watt

1. Introduction

1.1 Transfer RNA

Transfer ribonucleic acids (tRNAs) are low molecular weight RNA molecules, 75 to 95 nucleotides in length, that function as interpreters between the codons of the mRNA sequence and the amino acids used to produce the protein during translation (Rich and RajBhandary, 1976). Like most RNA molecules, tRNAs are not released from the transcription complex in a fully functional form (Schurer *et al.*, 2001), but instead must undergo several modifications. For example, 5' leader and 3' trailer sequences and any introns (if present) must be removed (Deutscher, 1984, Altman *et al.*, 1995, Deutscher, 1995, Morl and Marchfelder, 2001), numerous nucleotide residues must be modified (*e.g.*, to form T and ψ) (Bjork, 1995, Yokoyama and Nishimura, 1995) and finally, a 3'-terminal cytidine, cytidine, adenosine (CCA) sequence must be added, if it is not already present (Schurer *et al.*, 2001) (Figure 1). My research concentrated specifically on ATP (CTP): tRNA-specific tRNA nucleotidyltransferase (tRNA nucleotidyltransferase), the enzyme responsible for this CCA addition, so I will discuss this enzyme further.

1.2. ATP (CTP): tRNA-specific tRNA nucleotidyltransferase

All mature tRNA molecules have a CCA sequence at their 3' end (Schurer *et al.*, 2001). This sequence serves as the attachment site of the amino acid in protein synthesis. Without the CCA sequence neither aminoacyl-tRNA synthetases nor elongation factor Tu (Tamura *et al.*, 1994, Cusack, 1997, Liu *et al.*, 1998) can function. Furthermore, it has been shown that the CCA sequence is necessary for the exact positioning of the peptidyl-tRNA at the P site and the aminoacyl-tRNA at the A site on the large ribosomal subunit to facilitate peptide bond formation (Samaha *et al.*, 1995, Nissen *et al.*, 2000). Therefore, the CCA sequence plays an essential role in tRNA function.

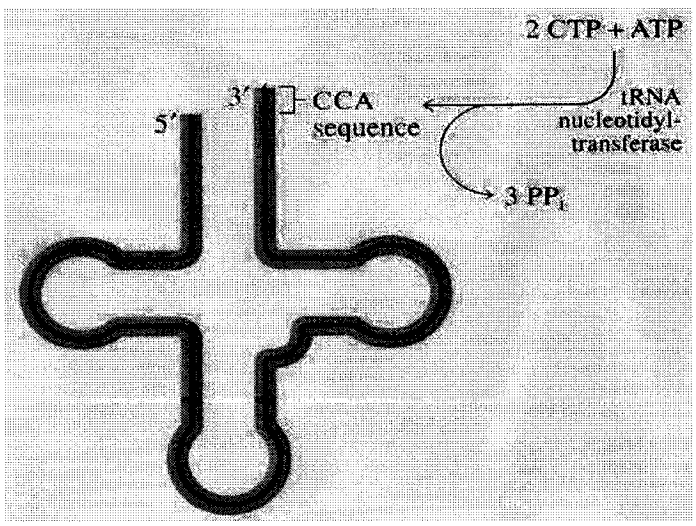


Figure 1: The role of tRNA nucleotidyltransferase (modified from Moran, 1994).

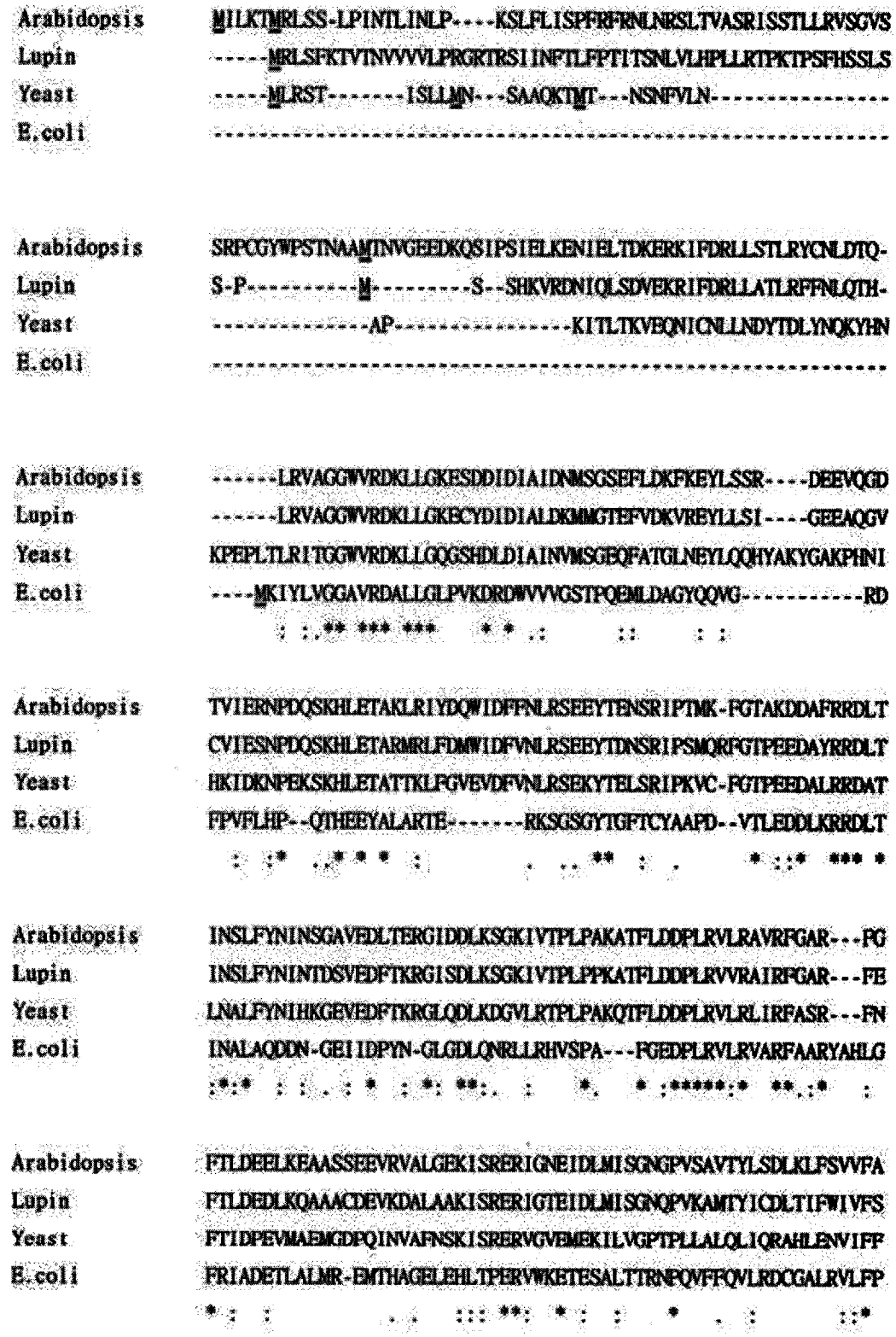
In eukaryotes, tRNA genes lack the CCA sequence such that it must be added post-transcriptionally (Morl and Marchfelder, 2001). Therefore, tRNA nucleotidyltransferase is essential in these organisms as has been shown to be the case in yeast (Giaever *et al.*, 2002). In *E. coli*, since the CCA sequence is already present in the primary transcript, tRNA nucleotidyltransferase is not essential (Zhu and Deutscher, 1987). However, without tRNA nucleotidyltransferase, cells grow more slowly suggesting a repair function for this enzyme in this organism (Deutscher *et al.*, 1974).

1.3 Intracellular localization of tRNA nucleotidyltransferase

In *E. coli,* tRNA maturation occurs in the single intracellular compartment defined by the plasma membrane. In contrast, eukaryotic cells are subdivided into functionally distinct, membrane-bound compartments or organelles. Protein synthesis can occur on ribosomes in the cytosol, mitochondrion and chloroplast. Therefore, tRNAs are needed in all of these locations. Like the nucleus, mitochondria and chloroplasts also contain tRNA genes (Martin, 1995) and these genes are transcribed to produce tRNAs that function in organellar protein synthesis. Therefore, in addition to the enzymes required for the production of functional tRNAs encoded by nuclear genes, there must be a complete set of proteins required for mitochondrial or chloroplast tRNA

maturation. Sequence analysis of numerous mitochondrial and chloroplast genomes (http://megasun.bchoumontreal.ca/ogmpproj.html) has revealed that as with most of the proteins required for organellar biogenesis, tRNA nucleotidyltransferase is not encoded on the organellar genome. Therefore, this protein must be imported into the organelles after translation in the cytosol of a transcript produced from a nuclear gene.

In yeast, neither the mitochondrial nor the nuclear tRNA genes encode the 3'terminal CCA sequence, so tRNA nucleotidyltransferase performs an essential function in tRNA biosynthesis in both of these locations. Using biochemical and genetic approaches it was demonstrated that the tRNA nucleotidyltransferase gene (*CCA1*) in *S. cerevisiae* codes for a sorting isozyme (Martin and Hopper, 1994), *i.e.*, the product(s) of a single gene function(s) in multiple cellular locations. A yeast strain carrying a temperature-sensitive mutation in the *CCA1* gene cannot grow at the non-permissive temperature (37°C) and CCA addition to both cytosolic and mitochondrial tRNAs is affected (Chen *et al.*, 1992), indicating that the products of this gene are required for CCA addition in the nucleus, cytosol and mitochondrion. Sequence analysis of the yeast *CCA1* gene revealed three in-frame ATGs at codon positions 1, 10 and 18 (underlined in Figure 2).


Arabidopsis
Lupin
Yeast
E.coli

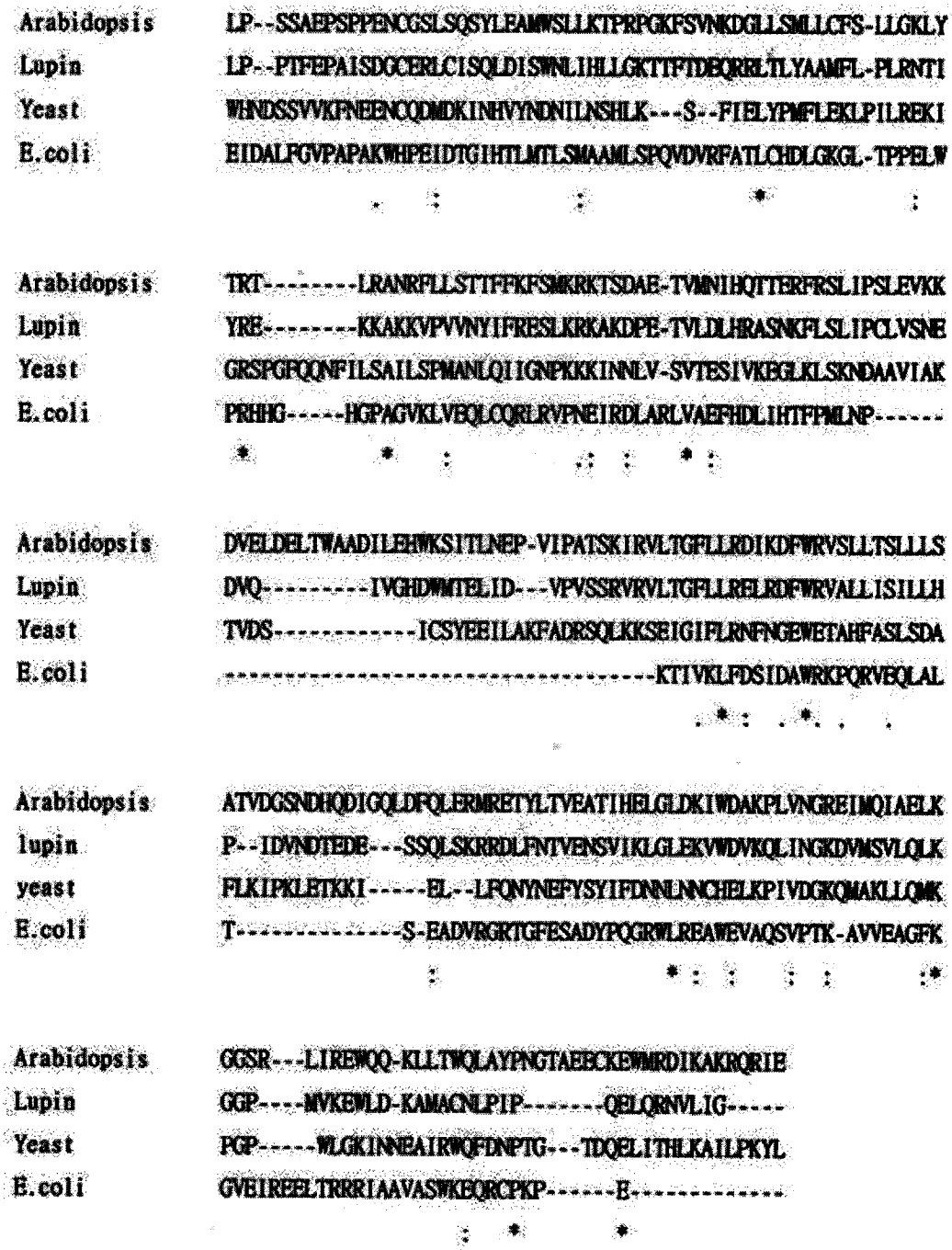


Figure 2: Multiple sequence alignment of tRNA nucleotidyltransferases from *Arabidopsis*, lupin, yeast, and *E. coli.*

Potential starting amino acids are underlined. Standard one letter abbreviations are used for the amino acids, "*" indicates an amino acid conserved in all four sequences, ":" indicates an amino acid strongly similar in the four sequences, "." indicates an amino acid weakly similar in the four sequences, and "-" indicates a gap introduced to optimize alignments.

When the full length gene (starting at ATG1) was used in complementation experiments with the temperature-sensitive strain, normal growth was seen on both fermentable and non-fermentable carbon sources. In contrast, when ATG1 and ATG10 were altered (such that protein synthesis initiates at ATG18), the cells could grow at the non-permissive temperature on glucose (a fermentable carbon source) but not on glycerol or lactose (non-fermentable carbon sources). Moreover, enzyme activity in mitochondria was eliminated (Chen *et al.*, 1992). Taken together these results suggest that the mitochondrial and nuclear/cytosolic forms of yeast tRNA nucleotidyltransferase are encoded by a single gene and that the first 17 amino acids are not essential for enzyme activity but are used as a mitochondrial targeting signal. This observation that an amino terminal sequence would serve as a mitochondrial targeting sequence is in good agreement with what is known about the general character of mitochondrial targeting signals (vonHeijne *et al.*, 1989).

Transfer RNA nucleotidyltransferase from lupin has been purified and characterized in our lab (Shanmugam *et al.*, 1996). As in yeast, the protein predicted from the cDNA encoding lupin tRNA nucleotidyltransferase contained additional amino-terminal sequences not present in the *E. coli* protein, and revealed two potential in frame start codons coding for amino acids 1 and 58 (Figure 2). Depending on which start codon is

used in translation, proteins produced will have, or lack, a 57 amino acid amino-terminal extension. These 57 amino acids have characteristics of both mitochondrial and chloroplast targeting signals (vonHeijne *et al.*, 1989), and are not essential for enzyme activity (Shanmugam *et al.*, 1996). This suggests that, as in yeast, the amino-terminal amino acids of the lupin enzyme encode an organellar (mitochondrial or chloroplast) targeting signal and that the lupin tRNA nucleotidyltransferase also represents a sorting isozyme. Analysis of the cDNA encoding the *Arabidopsis* tRNA nucleotidyltransferase (Gu, 2000), revealed a similar situation (Figure 2). Data on other fungal (Deng *et al.*, 2000, Hanic-Joyce and Joyce, 2002), plant (Shanmugam *et al.*, 1996, Sabetti, 2002) and animal (Nagaike *et al.*, 2001, Reichert *et al.*, 2001, Keady *et al.*, 2002) tRNA nucleotidyltransferases suggest that this might be a feature of all eukaryotic tRNA nucleotidyltransferases.

While work in our laboratory (Deng *et al.*, 2000, Hanic-Joyce and Joyce, 2002, Sabetti, 2002) is involved in identifying the targeting information contained on these proteins, we also are interested in determining whether other proteins are involved in directing sorting isozymes to their correct subcellular destinations. A number of different approaches can be used to identify proteins which interact with a protein of

interest, in this case tRNA nucleotidyltransferase. Some of the techniques that we have used to address this question will be discussed in the next section.

1.4. Protein-protein interactions

Protein-protein interactions are found in almost every biological process, *e.g.*, signal transduction (Simon *et al.*, 1991, Weiss, 1993), cell proliferation (Muller and Helin, 2000), cell cycle control (Lin *et al.*, 2002), DNA replication (Lopez de Saro and O'Donnell, 2001), transcription (Feaver *et al.*, 1993), translation (Palecek *et al.*, 2001), RNA splicing (Stojdl and Bell, 1999), secretion (Nicholas *et al.*, 2002) and programmed cell death (Sato *et al.*, 1994). Finding interactions between proteins involved in common cellular functions is a way to get a broader view of how these proteins work cooperatively in a cell. A number of biochemical, physical, genetic and microscopic techniques have been developed to detect specific protein-protein interactions, *e.g.*, co-elution (Ratner, 1974), coimmunoprecipitation (Lane and Crawford, 1979), far Western blotting (Blackwood and Eisenman, 1991, Kaelin *et al.*, 1992), yeast two-hybrid system (Fields and Song, 1989), bacterial two-hybrid system (Dove *et al.*, 1997), Biacore (Jonsson *et al.*, 1991), fluorescence resonance energy transfer (FRET) (Clegg, 1995), isothermal titration calorimetry (ITC) (Pierce *et al.*, 1999) and analytical

ultracentrifugation (AUC) (Rivas *et al.*, 1999). Examples of some of these techniques will be discussed.

1.4.1 Yeast two-hybrid system

The yeast two-hybrid system is a powerful tool for discovering protein-protein interactions *in vivo* (Fields and Song, 1989). It is based on the fact that a yeast transcription factor (Gal4p) consists of two physically separable domains: a DNA binding (DB) domain and a transcription activation (TA) domain. The DB domain targets the transcription factor to the specific upstream activation sequence (UAS) whereas the TA domain serves to initiate transcription of the downstream gene. Neither of these domains alone is able to activate transcription, but each domain continues to function when fused to other proteins.

In a yeast two-hybrid experiment, sequences encoding the two domains of the Gal4p transcription factor are cloned into two different vectors. The cDNA sequence coding for the protein of interest is fused to one domain, whereas cDNAs coding for potential interacting proteins, for example, from a cDNA library, are introduced into a vector containing the other domain. These two vectors then are co-transformed into a yeast strain containing a reporter gene under the control of the Gal4p UAS. If an

interaction between the two proteins of interest occurs, the DB and AT domains are brought into close proximity and a functional transcription activator is reconstituted, resulting in transcription of the reporter gene (Figure 3).

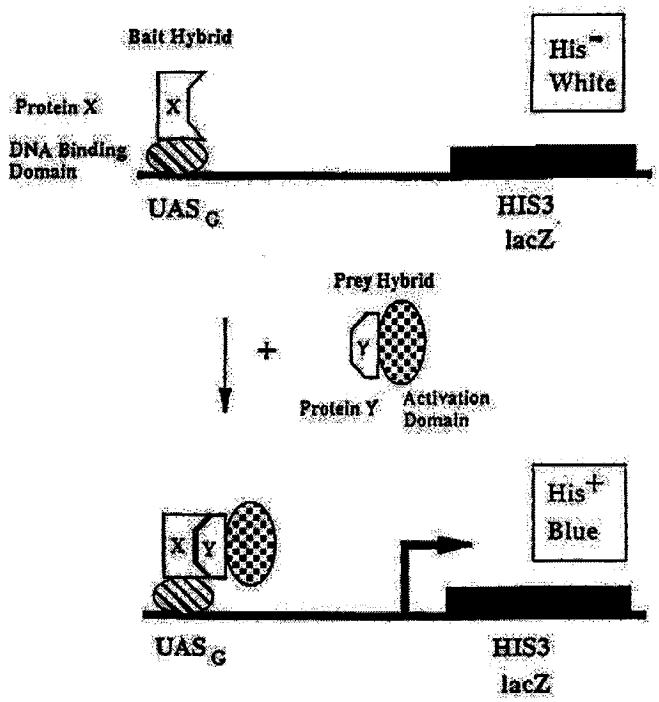


Figure 3: A schematic representation of the yeast two-hybrid system (Bai and Elledge, 1996).

One hybrid consists of protein X fused to the DNA binding domain while the other hybrid consists of protein Y fused to the activation domain. The DNA binding domain binds to the specific upstream sequence (UAS). Interaction between proteins X and Y brings the transcription activation domain to the UAS and activates expression of the reporter gene (*HIS3* or lacZ).

There are some limitations in using the yeast two-hybrid system to study protein-protein interactions. For example, real interactions may be missed because of a lack in yeast of other proteins normally required to stabilize the interaction (Phizicky and

Fields, 1995). There also may be competition with endogenous yeast factors (Estojak *et al.*, 1995) or the possibility that the fusion protein is not produced or folded correctly in yeast cells (Phizicky and Fields, 1995). Moreover, the interactions detected by this method might not have biological significance as the yeast nucleus may not represent the natural environment of many proteins. In addition, very-low-affinity interactions with Kd on the order of 1 μM will not be detected (Golemis and Brent, 1992, Estojak *et al.*, 1995). Conversely, false positives may arise if bait proteins activate the transcription of the reporter gene by themselves (auto-activation) (Estojak *et al.*, 1995). Therefore, detected interactions need to be confirmed by using a second assay system. For example, additional *in vivo* methods may include the bacterial two-hybrid system, or co-localization using GFP or antibodies to the proteins of interest. *In vitro* methods such as co-immunoprecipitation, chemical cross-linking, co-elution or far Western blotting also may be used.

1.4.2 Co-elution

Co-elution is based on the fact that a protein of interest can be coupled to a matrix under controlled conditions and used through column chromatography as a bait protein to select a ligand protein in an appropriate mixture of proteins (Phizicky and Fields, 1995).

For example, a glutathione *S*-transferase (GST) fusion protein could be retained on Glutathione Sepharose resin, or a histidine-tagged fusion protein could be retained on a metal affinity resin (Figure 4A). Subsequently, a mixture of proteins could be added to the column. If a component of the mixture interacts with the protein already bound to the column then it will be retained as well. If there is no interaction it will not be retained (Figure 4B). If the two proteins can be eluted together this suggests that an interaction has occurred (Figure 4C).

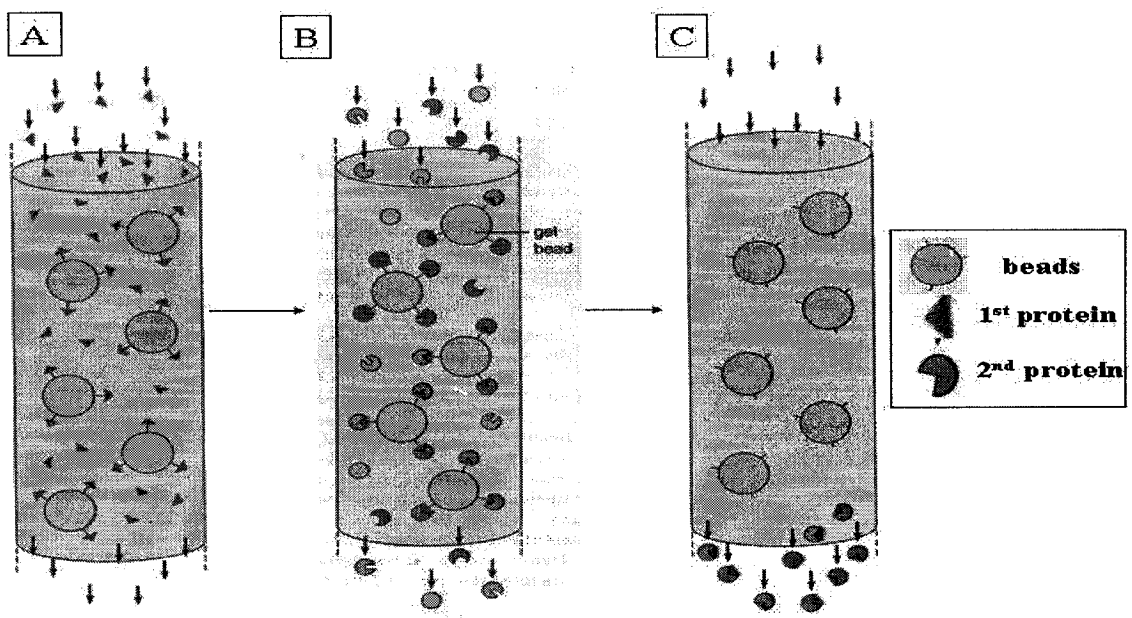


Figure 4: Co-elution (derived from Wolfe, 1995)

A) Protein containing an appropriate tag for binding is loaded into the affinity column. B) The second protein or a mixture or proteins is added to the column. If any proteins in this mixture interact with the protein bound on the column, they will stay on the column too; otherwise, they will flow through. C) The bound proteins are eluted together and analyzed.

Even though co-elution is a sensitive technique and can detect proteins interacting with binding constants around 10^{-5} M (Formosa *et al.*, 1991), some considerations such as whether the protein can be maintained in its native state (Greenblatt and Li, 1981) or carries the appropriate modifications (Felder *et al.*, 1993, Ludlow *et al.*, 1993) need to be considered in this type of *in vitro* assay. Appropriate control experiments must be carried out to eliminate false positives or false negatives. For example, to make sure that the bait protein can bind to the column and that the prey protein can bind to the column only when the bait protein is present. There are several possible explanations for false-positive signals when using this method, *e.g.*, proteins might interact with high specificity even though they never encounter one another in the cell (Lazarides and Lindberg, 1974). False negatives might arise in a situation where interactions require other components that are not present or the two proteins may not be able to interact with each other while bound to the resin.

1.4.3 Far Western blot

The far Western blot (also called a west Western or a ligand blot) has been used widely to examine the interactions of many diverse proteins. For example, it has been used to examine the interactions between the subunits of eukaryotic initiation factors

(Kimball *et al.*, 1998), the interactions between basic helix-loop-helix DNA-binding proteins (Chaudhary *et al.*, 1997), and the interactions between keratin intermediate filaments and desmosomal proteins (Kouklis *et al.*, 1994). This method is useful when proteins are difficult to solubilize or extract from cells, or when proteins cannot be expressed in bacteria or yeast due to toxicity problems (Edmondson and Dent, 2001). It is a flexible method and proteins prepared in a variety of ways can be used for the assay. Cell extracts, recombinant proteins and peptides all can be used as either probe or target proteins. In addition, many different detection techniques can be used. The protein of interest can be radioactively labeled, biotinylated or used in the blotting procedure as an unlabeled probe that is detected by a specific antibody. Finally, the far Western can be modified to define the protein domains and amino acid residues that are important in protein-protein interactions (Edmondson and Dent, 2001).

In a far Western, one protein of interest can be fractionated by polyacrylamide gel electrophoresis (PAGE) (Figure 5A) and transferred to a nitrocellulose membrane (Figure 5B). The protein immobilized on the membrane (Figure 5C) then can be verified with a library used to identify a protein, peptide or ligand that will bind to it. This method is similar to immunoblotting except that in the case of a far Western the antibody only binds to the second protein or probe (Figure 5D - 5F). An advantage of this technique is that

complex mixtures of proteins, such as total-cell lysates, can be analyzed without any purification. Cell lysates also can be fractionated before gel electrophoresis to increase the sensitivity of the method for detecting interactions with rare proteins.

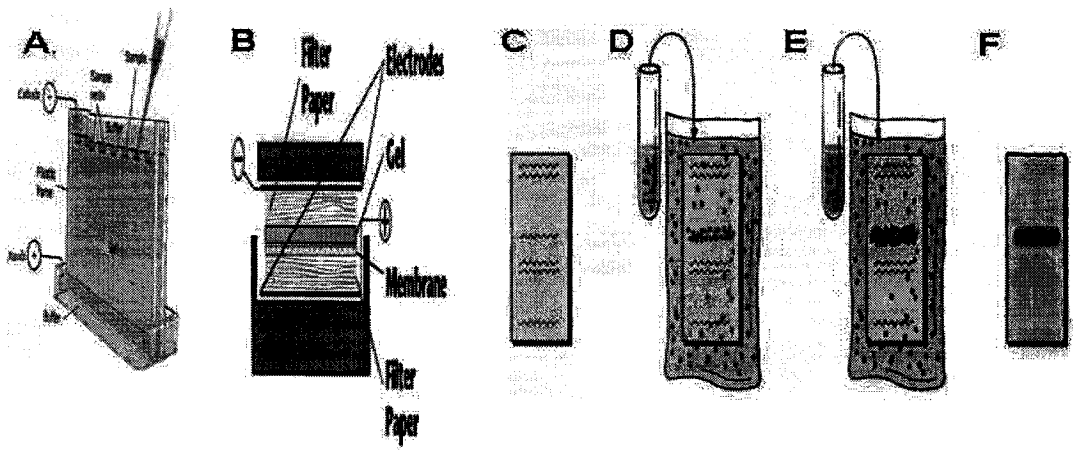


Figure 5: The detection of protein interactions by far Western blot (derived from Voet and Voet, 1995).

A) A mixture of proteins, including the protein of interest, is applied to SDS-polyacrylamide gel electrophoresis. The proteins migrate at differing rates according to their respective sizes. B) The proteins are transferred from the gel onto a membrane. C) Unoccupied binding sites on the membrane are blocked. D) The blot is incubated with the "prey" protein. E) The membrane is incubated with antibody to the prey protein. Unbound antibody is washed away and incubated with an enzyme-linked antibody specific for the primary antibody. F) Any interactions are seen by assaying the linked enzyme.

Even though far Western blotting is a very flexible method, some care is still required in interpreting the results. False negatives may result because of the denaturing gels used. SDS-PAGE will inactivate most proteins and separate subunits of a complex. Although denaturants are removed during the blotting procedure allowing many proteins

to recover or partially recover their activity (Phizicky and Fields, 1995), if biological activity is not recoverable, this might result in a false negative. In some cases, if the probe protein is excessively degraded, a mixture of different products are generated (Einarson and Orlinick, 2002). This may result in variable results from different fusion protein preparations and could cause a false positive.

1.4.4 Cross-linking

Cross-linking is a technique for covalently linking distinct chemical functionalities. In the case of proteins, either nucleophilic side chains or the termini of the polypeptide chains are the functional groups that are linked (Nadeau and Garlson, 2002). Cross-linking can be used to study both the interactions of proteins within a stable complex or those that interact transiently. Protein cross-linking is a widely used method of determining near-neighbor relationships of proteins, three-dimensional structures of proteins, enzyme-substrate orientation and molecular associations in cell membranes (Nadeau and Garlson, 2002).

Many factors must be considered to obtain optimal cross-linking for a particular application. Factors that affect protein folding (*e.g.*, pH, salt, additives and temperature)

may alter conjugation results. Other factors such as protein concentration, cross-linker concentration, number of reactive functional groups on the surface of a protein, cross-linker spacer arm length and conjugation buffer composition also must be considered (Nadeau and Garlson, 2002).

One of the cross-linkers used in this experiment was glutaraldehyde. Glutaraldehyde is a commonly used homobifunctional, dialdehyde cross-linker. The cross-linking reaction proceeds through a Schiff base to an irreversible product (Wong, 1991). When the reaction is carried out at neutral or slightly alkaline pH, α, β-unsaturated aldehyde polymers of glutaraldehyde are formed. The unsaturated polymer then cross-links to the amino groups of proteins as illustrated in Figure 6. The interaction of the Schiff base with adjacent double bonds provides stability against hydrolysis. With excess amino groups, nucleophilic addition of the ethylenic double bond is possible. Because of its different polymeric forms, the distance between two cross-linked groups cannot be estimated. Glutaraldehyde is most often used to cross-link and stabilize protein crystals as well as proteins in solution (Wong, 1991).

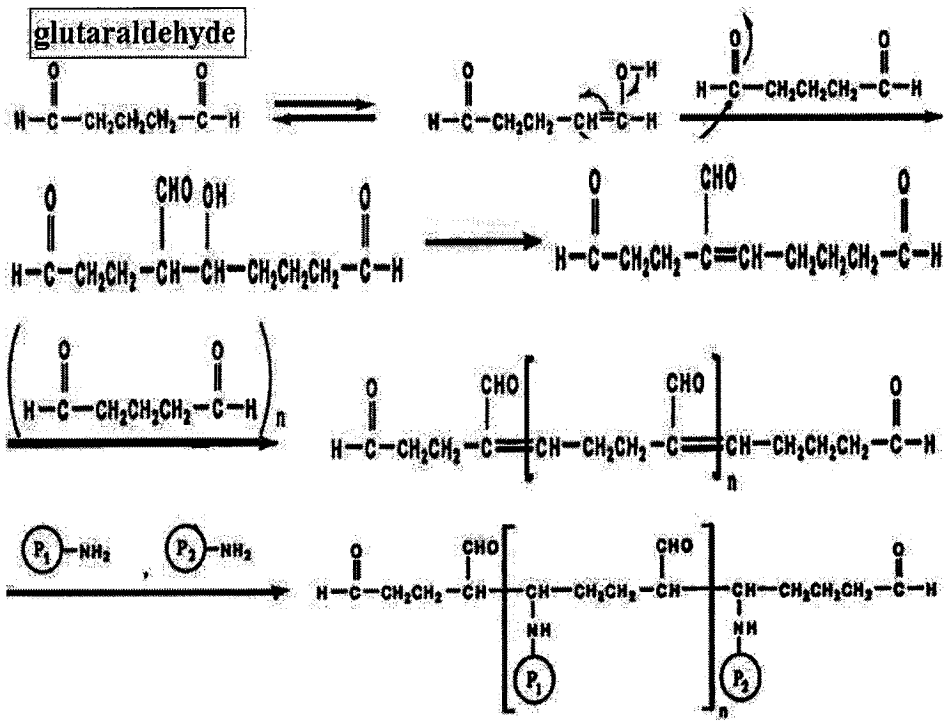


Figure 6: Polymerization of glutaraldehyde and its cross-linking to proteins (Wong, 1991).

The cross-linking reaction of glutaraldehyde is first to form α, β-unsaturated aldehyde polymers, which then cross-link to the amino groups of proteins.

A second cross-linking agent, EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimde), belongs to the zero-length cross-linker group. Zero-length cross-linking reagents activate side-chain functional groups on targeted proteins, allowing covalent bonds to form between the side chains without insertion of an exogenous spacer (Nadeau and Garlson, 2002). Since no spacer is introduced, zero-length cross-linking provides a better chance that linked residues are located in the actual site(s) of protein-protein interaction. The EDC activates carboxylate groups to form active O-acrylisourea

intermediates between the reagent and the carboxyl group of glutamic or aspartic acid, followed by a nucleophilic substitution with an amino group (lysine) (Wong, 1991). The problem with the EDC cross-linking reaction is O-acrylisourea derivatives can undergo rapid hydrolysis. Therefore, the activated carboxyl group may be deactivated before the complex has time to form. To stabilize the O-acylisourea derivative, NHS (N-hydroxysuccinimide) or "active esters" are added. Typically, one component of the complex is activated with EDC in the presence of NHS, leading to the formation of N-succinimidyl esters. Subsequently, the activation step is terminated by the addition of β–mercaptoethanol which rapidly reacts with excess EDC to prevent activation of components of the complex other than that initially activated. Finally, the other component of the protein complex is added to complete the cross-linking reaction (Figure 7) (Grabarek and Gergely, 1990).

The main problem with cross-linking techniques is that they detect nearest neighbors which may not be in direct contact. If one cross-linking agent fails to detect the interaction of two proteins, it could mean either that the proteins do not interact or that no functional group is nearby.

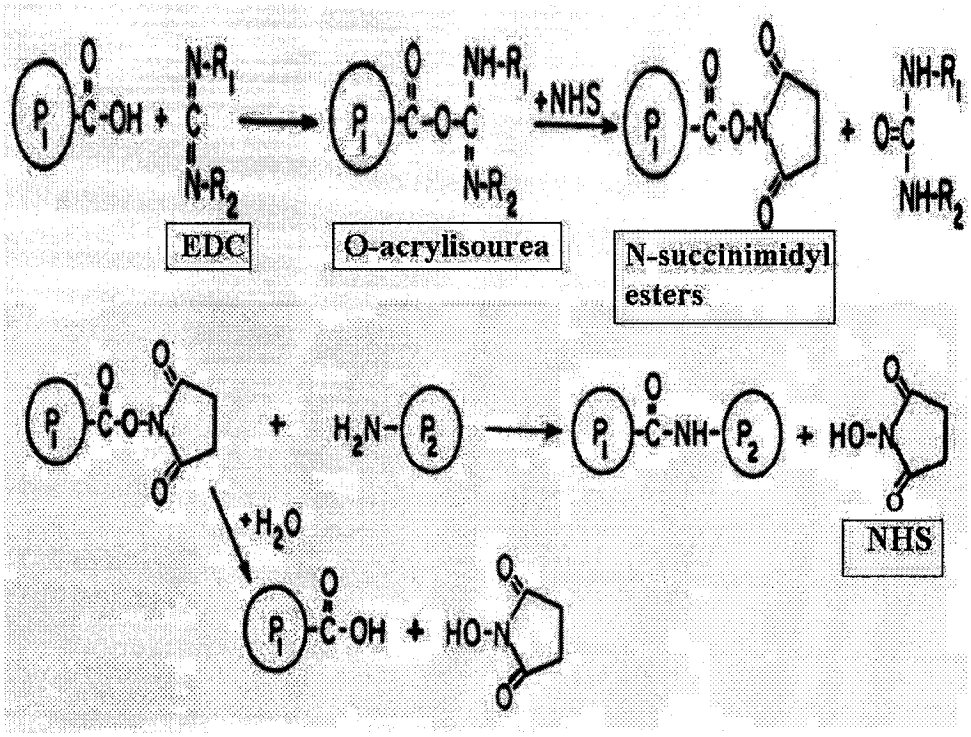


Figure 7: Cross-linking of proteins by N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimde (EDC) (Grabarek and Gergely, 1990).

The Zero-length cross-linking reaction is initiated by adding protein 1 (P1) in the presence of EDC and NHS to form N-succinimidyl ester. Protein 2 (P2) then is added into the activated complex to form the cross-linking product.

1.5. Proteins identified by yeast two-hybrid assay

In our lab, using the yeast two-hybrid system, several *Arabidopsis* proteins have been identified that interact with either lupin or *Arabidopsis* tRNA nucleotidyltransferase (Gu, 2000). The *Arabidopsis* homologue of the yeast *YML079w* gene product (AraYml079wp) encoded by the *Arabidopsis* At1g19130 gene was identified as interacting with the lupin tRNA nucleotidyltransferase (Gu, 2000). The *Arabidopsis*

homologues of the yeast *GIM1* gene product (AraGim1p) and the yeast kinesin light chain, encoded by the *Arabidopsis* At1g29990 and At3g27960 genes, respectively, showed interactions with the *Arabidopsis* tRNA nucleotidyltransferase (Gu, 2000). In each of these cases, the interaction was dependent on the presence of the amino-terminal organellar targeting signal of tRNA nucleotidyltransferase, *i.e.*, fusion proteins lacking these targeting signals did not generate positive interactions (Gu, 2000). This suggested that these interacting proteins may play some role in the targeting of tRNA nucleotidyltransferase. Two of these proteins were the subject of further study here.

1.5.1 *Arabidopsis* homologue of the yeast *GIM1*gene product (AraGim1p)

AraGim1p is a 129 amino acid polypeptide (Gu, 2000). The predicted protein sequence shows 33% sequence identity (with e value of 0.014) over its complete length to the 114 amino acid yeast Gim1p (GeneBank Accession No. CAA54062) (Figure 8).

The primary sequences of these proteins are conserved among many other eukaryotes including human, mouse, *C. elegans* and *S. pombe* (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). This high sequence conservation suggests a common function for this protein in all of these organisms. Experiments (Geissler *et al.*, 1998) showed that the yeast Gim1p promotes the formation of functional

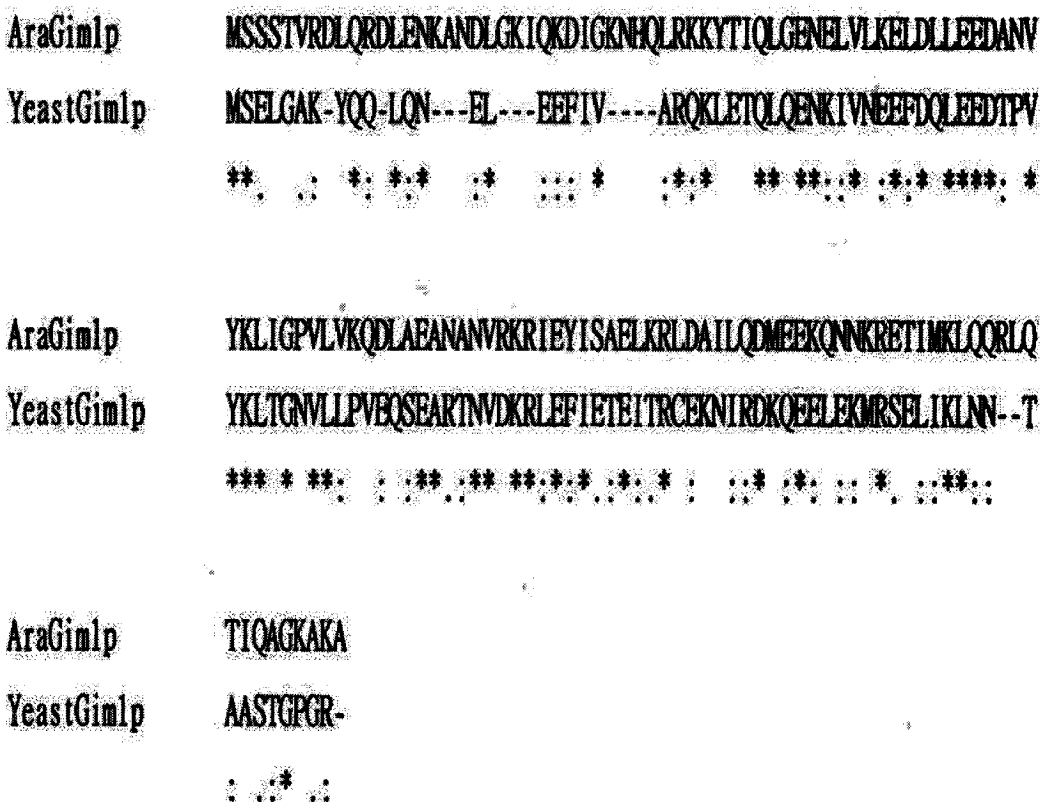


Figure 8: Amino acid sequence similarity of the *Arabidopsis* and yeast Gim1 proteins. Standard one letter abbreviations are used for the amino acids, identical amino acids in the two sequences are indicated "*", ":" indicates strongly similar amino acids in the two sequences, "." indicated weakly similar amino acids and "-" indicates a gap introduced to optimize alignments. Numbers indicate the amino acid positions.

α and γ-tubulin, the subunits of microtubules. It may function in protein folding and assembly processes, possibly as a molecular chaperone (Geissler *et al.*, 1998). The yeast *GIM1* gene product belongs to the *GIM* (Genes Involved in Microtubule biogenesis) complementation group. Deletion of the *GIM1* gene causes microtubule defects in yeast cells, resulting in a cold-sensitive growth defect, increased sensitivity toward the

microtubule-depolymerizing drug benomyl and synthetic lethality with a mutated yeast γ-tubulin (Geissler *et al.*, 1998). The cold and benomyl-sensitive phenotypes were complemented by the AraGim1p, indicating that the function of the *GIM1* gene product is conserved between yeast and plants (Sabetti, 2002). Molecular interaction data from organism-wide screens of *Saccharomyces cerevisiae* (http://biodata.mshri.on.ca/grid/servlet/SearchResults?keywords=YLR200W) suggest that the yeast Gim1p interacts with one hundred and sixty-six proteins including actin binding proteins, tubulin binding proteins, DNA binding proteins, structural constituents of the cytoskeleton and chaperones, but not the yeast tRNA nucleotidyltransferase.

1.5.2 *Arabidopsis* homologue of the yeast *YML079w* gene product (AraYml079wp)

AraYml079wp is predicted to contain 188 amino acids (Gu, 2000). This protein is encoded by the At1g19130 gene located on *Arabidopsis* chromosome 1. When the predicted amino acid sequence of this protein was used to search GenBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi BLASTP 2.2.7 [Jan-05-2004], RID: 1073535068-21463-93299433326.BLASTQ4), forty six proteins showing sequence similarity were identified. All of these are hypothetical or uncharacterized proteins.

Only five of these proteins were from eukaryotes other than *Arabidopsis* and one of these was from *Saccharomyces cerevisiae*. The At1g19130 gene product shows 26% sequence identity to the yeast *YML079w* gene product (GenBank Accession No. S48818) (Figure 9).

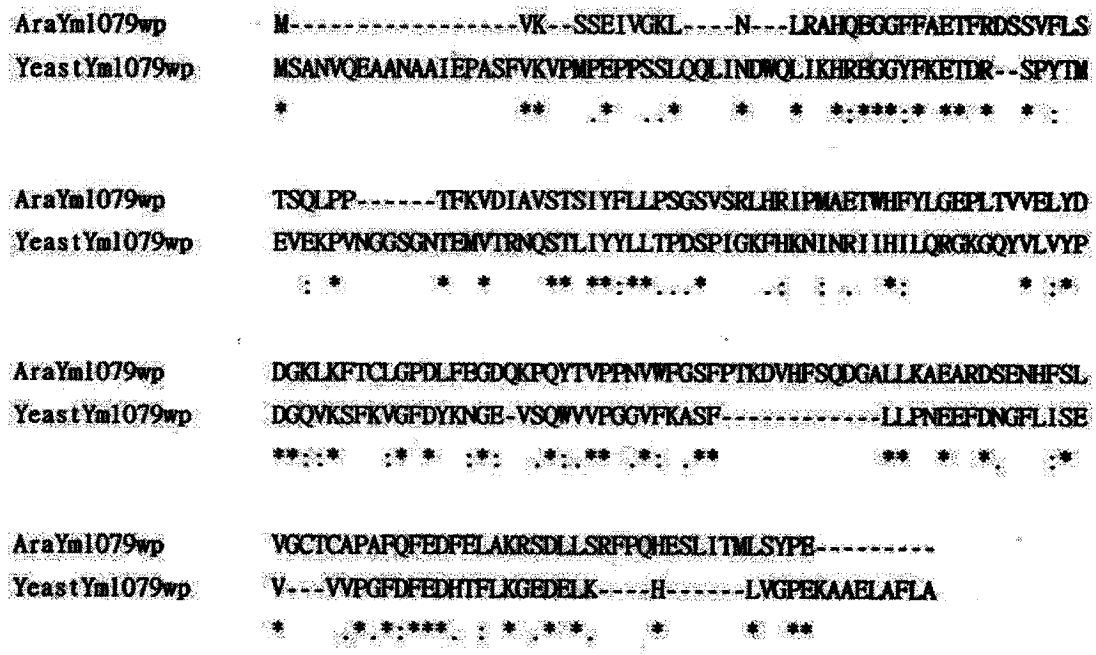


Figure 9: Amino acid sequence similarity of AraYml079w and yeast *YML079w* gene products.

Standard one letter abbreviations are used for the amino acids, identical amino acids in the two sequences are indicated "*", ":" indicates a strongly similar amino acids in the two sequences, "." indicated weakly similar amino acids, and "-" indicates a gap introduced to optimize alignments. Numbers indicate the amino acid positions.

This protein has no known function. Gene deletion experiments in yeast have shown that this gene is not essential (Giaever *et al.*, 2002). Molecular interaction data from an organism-wide screen of *Saccharomyces cerevisiae* suggest that the *YML079w*

gene product interacts with the yeast *ATP14* gene product but not with the yeast tRNA nucleotidyltransferase (Ito *et al.*, 2001). Intriguingly, the *ATP14* gene product also contains a mitochondrial targeting signal (Arselin *et al.*, 1996).

1.6. This work

In a previous study using the yeast two-hybrid system, results suggested that AraYml079wp interacted with the lupin tRNA nucleotidyltransferase and AraGim1p showed an interaction with *Arabidopsis* tRNA nucleotidyltransferase. In this study, several biochemical methods were used to attempt to confirm the interactions suggested by the yeast two-hybrid data. In addition, an attempt was made to define the role of Yml079wp by studying, under a number of different experimental conditions, a yeast strain that had the gene coding for this protein deleted.

2. Materials and methods

2.1. Strains and growth media

Saccharomyces cerevisiae strains BY4743ΔYDL227c (HO), BY4743ΔYKR066c (CCP1) and BY4743ΔYML079w from the BY4743 deletion strain set (Giaever *et al.*, 2002) were used in the characterization of the AraYml079wp homologue. The yeast strains are homozygous diploids generated by the *Saccharomyces* Genome Deletion Project, distributed by Research Genetics, and were kindly provided by Alain Bataille. Yeast strain SGY101 p415-ADH, provided by Antonino Sabetti, was used as a positive control for cold sensitivity. Yeast strain NT33-5, provided by Dr. Pam Hanic-Joyce, was used as a positive control for temperature sensitivity. The genotypes of these strains are listed in Table 1. *Escherichia coli* strain XL2-Blue was purchased from Stratagene. Growth media used in this work are listed in Table 2.

Table 1: Strains used in this work

Strain	**Organism**	**Genotype**	**Reference**
XL2-Blue	***E. coli***	*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *relA*1 *supE*44*lac*(*F'proAB*$^{+}$ *lacI*^{q}ZΔ*M*15 Tn10 Tet^{r} Amy Cam^{r})^{a}	Stratagene catalog 1999
NT33-5	***S. cerevisiae***	Relevant genotype *cca1-1*	Shanmugam *et al.*, 1996

SGY101	***S. cerevisiae***	*MAT α ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1lyke2::kanMX4*	Geissler *et al.*, 1998
BY4743	***S. cerevisiae***	*MATa/a his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0lys2Δ0/LYS2 MET15/met15D0 ura3D0 /ura3Δ0* (4741/4742)	Brachmann *et al.*, 1998

Table 2: Growth media

Growth medium	**Recipe**	**References**
YT	0.8% Bacto-tryptone 0.5% Bacto-yeast extract 0.5% NaCl (plates: 1.5% agar)	Sambrook *et al.*, 1989
Luria Bertani (LB)	1% Bacto-tryptone 0.5% Bacto-yeast extract 1% NaCl	Sambrook *et al.*, 1989
YPD	1% Bacto-yeast extract 2% peptone 2% dextrose (plates: 2% agar)	Guthrie and Fink, 1991
YPG	1% Bacto-yeast extract 2% peptone 3% glycerol (plates: 2% agar)	Guthrie and Fink, 1991

2.2. Plasmids

Two commercial plasmids, pGEX2T (Amersham) and pTrcHis (Invitrogen), were used in this project for heterologous protein expression in *E. coli.* The vector pGEX2T (Figure 10) contains the gene coding for glutathione S-transferase (GST) followed by a multiple cloning site. Expression of a GST fusion protein is under the control of the inducible *tac* promoter. A thrombin recognition site for cleaving the desired protein from the GST protein is present.

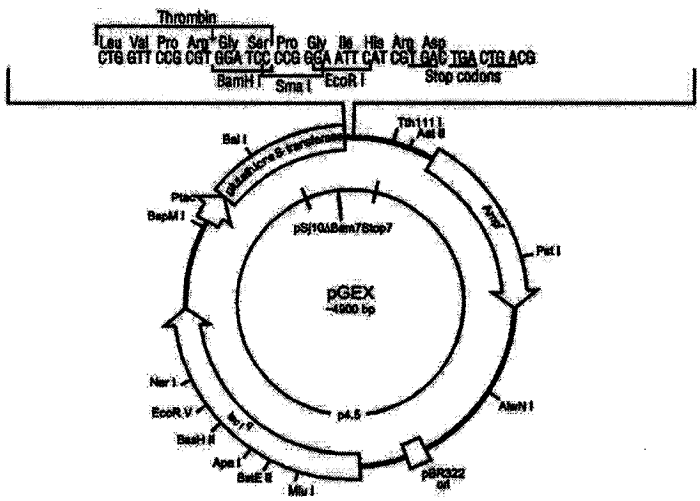


Figure 10: Map of plasmid pGEX2T (http://www4.amershambiosciences.com).

The pTrcHis vector (Figure 11) has a poly-His tag attached at the beginning of the multiple cloning site. Expression of the fusion protein can be induced under the control

of the *trc* promoter. Enterokinase can be used to cleave the His-tag from the protein of interest.

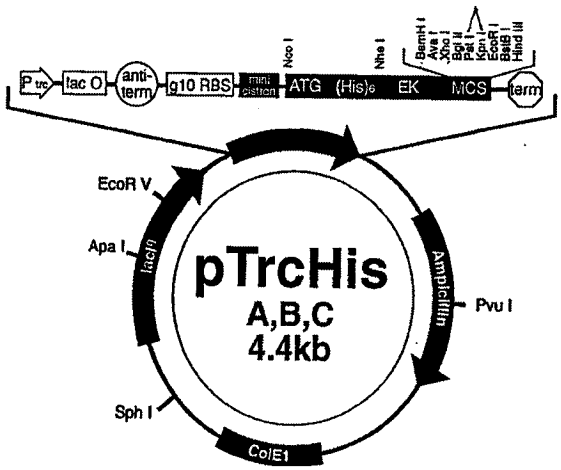


Figure 11: Map of plasmid pTrcHis (Invitrogen catalogue).

The cDNAs coding for the *Arabidopsis* At1g29990 and At1g19130 genes (coding for the homologues of the yeast *GIM1* and *YML079w* genes, respectively) were transferred into pTrcHis (Invitrogen) and transformed into XL2 Blue by Laura Weir. The pGEX2TlupinCCA1 and pGEX2T*ara*CCA1 plasmids were constructed by Jun Gu (Gu, 2000). Two additional plasmids, pGEX2TlupinCCA2 and pGEX2T araCCA3 expressing tRNA nucleotidyltransferases without their amino-terminal targeting signals,

were generated in this study as described below. The plasmid derivatives used in the fusion protein experiments are summarized in Table 3.

Table 3: Plasmid derivatives used for expression in the heterologous system

Plasmid	Promoter for fusion protein	Tag	Gene of interest	Restriction sites used	Protein product
pGEX2TaraCCA1	Tac	GST	*Arabidopsis* CCA gene from 1st ATG	*Eco*RI	AraCCA1
pGEX2TaraCCA3	Tac	GST	*Arabidopsis* CCA gene from 3rd ATG	*Eco*RI	AraCCA3
pGEX2TlupinCCA1	Tac	GST	Lupin CCA gene from 1st ATG	*Sma*I	lupinCCA1
pGEX2TlupinCCA2	Tac	GST	Lupin CCA gene from 2nd ATG	*Sma*I	lupinCCA2
pTrcHisKE2	Trc	$(His)_6$	AraGim1p	*Eco*RI and *Bam*HI	AraGim1p
pTrcHis108	Trc	$(His)_6$	AraYml079wp	*Eco*RI and *Bam*HI	AraYml079wp

2.3. Plasmid construction

2.3.1 Polymerase chain reaction (PCR)

In order to generate the lupin tRNA nucleotidyltransferase lacking its first 57 amino acids, and the *Arabidopsis* tRNA nucleotidyltransferase lacking its first 68 amino

acids, the polymerase chain reaction was used. Templates, primers and restriction sites (underlined) used for this amplification are listed in Table 4.

Table 4: Primers designed for polymerase chain reaction (PCR).

Primer	Sequence	Use	Template
ARAATG3NE	5'-CAT ATG GGA ATT CGA **ATG** ACG AAT GTT GGA GAG G-3'	5' primer for araCCA3	pzL1*Ara*CCA (provided by Jun Gu)
CCAARA3'	5'-CGG AAT TCG TCG ACA ATG **TTA** GTG G-3'	3' primer for araCCA3	pzL1*Ara*CCA (provided by Jun Gu)
LUP2GEX	5'- TCT CCC GGG ACA T**AT G**TC TTC ACA CAA GG-3'	5' primer for lupinCCA2	Lupin 1D1 (provided by Jun Gu)
LUP3'SS	5'- TCC CCC GGG TCG AC**T CA**A CCA ATC AAG -3'	3' primer for lupinCCA2	Lupin 1D1 (provided by Jun Gu)

Start and stop codons are in bold. Restriction sites *Sma*I (CCCGGG) and *Eco*RI (GAATTC) are underlined.

The PCR reactions were performed in a PERKIN ELMER DNA thermal cycler. The reaction mixture contained 100 pmol of each primer, 10 ng of template DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 U of Pfu DNA polymerase (Stratagene) and 1X Pfu polymerase buffer in a final volume of 100 μl adjusted with water. The mixture was

overlaid with 100 µl of mineral oil to avoid evaporation at high temperature. The enzyme was added after the PCR reaction was heated to 96°C for 1.5 minutes. This was followed by 30 cycles of denaturation at 96°C for 30 seconds, annealing at 40°C for 1 minute, and extension at 72°C for 4 minutes. The last cycle was finished at 72°C for 10 minutes to ensure complete extension of products.

2.3.2 Ethanol precipitation of PCR products

After 5 µl of each PCR reaction mix had been set aside for agarose gel electrophoresis, the remaining 95 µl were transferred to an Eppendorf tube containing two volumes of 99% ethanol and 1/10 volume of sodium acetate. The samples were placed at -80°C for 30 minutes and centrifuged at 14 000 rpm in an Eppendorf microfuge for 30 minutes at 4°C. The pellet was washed with 70% ethanol, desiccated and resuspended in 10 µl of TE (10 mM Tris [pH8], 1 mM EDTA).

2.3.3 Plasmid preparation

Plasmid DNA was isolated from *E. coli* cells following the alkaline lysis procedure of Good and Nazar (1997) with minor modifications. LB medium (Table 2) containing ampicillin (50 mg/l) was inoculated with XL2-Blue cells transformed with the

appropriate vector. The cultures (5 ml) were grown at 37°C overnight and collected by centrifugation of 1.5 ml aliquots for 5 seconds in Eppendorf tubes. The pellets were resuspended in 200 µl of GTE (50 mM glucose, 25 mM Tris [pH8], 10 mM EDTA [pH8]) by vortexing, 0.5 µl of RNase A (10 mg/ml) were added and each sample was set at room temperature for 10 minutes. Subsequently, 300 µl of 0.2 M NaOH, 1% sodium dodecyl sulfate were added, the samples were mixed by inversion and left at room temperature for 4 minutes. Next, 230 µl of 3 M sodium acetate (pH 5.2) were added and the samples were mixed by inversion and incubated on ice for 10 minutes. The tubes then were centrifuged for 20 minutes at 14 000 rpm at 4°C. The supernatants were collected and 200 µl of 30% polyethylene glycol 8000 were added. The samples were mixed by inversion and set on ice for 1 hour. The tubes then were centrifuged for 10 minutes at 14 000 rpm at 4°C. The supernatant was discarded, 1 ml of 80% ethanol was added and the samples were centrifuged for 5 minutes at 14 krpm at 4°C. The supernatant was again discarded, the samples were desiccated for 20 minutes and the pellets were resuspended in 20 µl of sterile TE.

2.3.4 Restriction digestion

The restriction digestion mix contained 2 μg of PCR products or plasmids, 1μl of 10X buffer Y with BSA (MBI) for *Sma*I digestion or 10X buffer E (Promega) for *Bam*HI digestion or for *Eco*RI digestion, 2-5 units of *Sma*I (MBI) or *Bam*HI (Promega) or *Eco*RI (Promega) and water to make 10 μl final volume. The samples were incubated for 2 hours at 30°C for *Sma*I, or at 37°C for *Bam*HI and *Eco*RI. For the plasmid, after 1.5 hours, 1 unit of CIAP (calf intestinal alkaline phosphatase, MBI) was added to the digestion mix. The digestion products were separated by agarose gel electrophoresis (described below) and the appropriate fragments were excised.

2.3.5 Agarose gel electrophoresis

Samples were separated on agarose gels, 1% agarose in TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, 1 μg/ml ethidium bromide) or 0.7% agarose in TEA buffer (4.84 g/l Tris, 1.14 ml/l glacial acetic acid, 1 mM EDTA (pH 8), 1 μg/ml ethidium bromide) by electrophoresis at 80 volts for about 2 hours.

2.3.6 Fragment purification

DNA fragments were purified from agarose gels by the phenol freeze-fracture method (Bewsey *et al.*, 1991). A piece of the agarose gel containing the DNA fragment of interest was cut out and crushed in a 1.5 ml Eppendorf tube. Phenol (400 µl) was added to this tube which was vortexed vigorously and placed at -80°C for 30 minutes followed by thawing at 37°C for 30 minutes. Another 400 µl of phenol was added, the sample was vortexed, placed again at -80°C for 30 minutes, and thawed at 37°C for 30 minutes. Then, 250 µl of water and 50 µl of cold 3M sodium acetate were added, the sample was vortexed and centrifuged at 14 000 rpm for 15 minutes at 4°C. The aqueous phase was collected, an equal volume of phenol was added, and the sample vortexed and centrifuged for 4 minutes at 14 000 rpm at 4°C. The aqueous phase was collected and the phenol extraction process was repeated. The final aqueous phase was extracted twice with equal volumes of ether. To the final aqueous phase was added 2 volumes of 99% ethanol and ethanol precipitation was carried out as described previously. The final pellet was resuspended in 10 µl of sterile TE by vortexing.

2.3.7 Ligation

The PCR products were inserted into the *Sma*I site (for the lupin gene) or the *Eco*RI site (for the *Arabidopsis* gene) within the multiple cloning site of pGEX2T. Insert (100-500 ng) and plasmid (10-60 ng) were mixed with 1X ligase buffer (MBI), 2.5 units of ligase (MBI) and 0.25 mM ATP in a final volume of 20 µl and incubated at 4°C overnight. Unligated (linear) vector and ligated dephosphorylated vector (no insert) were used as controls.

2.3.8 *E. coli* transformations

E. coli transformations were based on a modification of the protocol described by Capage and Hill (1979) and Lederberg and Cohen (1974). Transformations were achieved by mixing, in a sterile Eppendorf tube, 5 µl of ligation mixture with 50 µl of competent XL2 Blue cells (prepared by Dr. Pam Hanic-Joyce). The mixture was left on ice for 20 minutes and the transformed cells were plated on warmed LB agar plates containing ampicillin (50 µg/ml) and incubated overnight at 37°C.

2.3.9 Screening for transformed cells

The screening process was adapted from the rapid screening procedure of Promega with minor modifications. The transformed cells were selected and patched onto LB/AMP plates and incubated at 37°C overnight. Patched cells were smeared in microcentrifuge tubes and resuspended in 50 µl of 10 mM EDTA (pH 8) and 50 µl of cracking buffer (0.2 M NaOH, 20% sucrose and 1%SDS) by vortexing. The mixture was incubated at 70°C for 5 minutes. Before incubating on ice for 5 minutes, 1.5 µl of 4 M KCl and 1 µl of 0.4% bromophenol blue were added to the tubes. Prior to loading, samples were centrifuged at 14 000 x g for 3 minutes at 4°C. The samples were electrophoresed through a 0.7% agarose 1X TEA gel described in the previous section. Control cells containing plasmid without insert were used as size markers. Insert size and orientation were confirmed by plasmid isolation and restriction digestion (*Bam*HI) and gel electrophoresis as described previously.

2.4. Protein-protein interactions

2.4.1 Over-expression of fusion proteins

E. coli cells for isolation of lupin or *Arabidopsis* tRNA nucleotidyltransferase (with or without the chloroplast or mitochondrial targeting signal); AraGim1p and

AraYml079wp were cultured in 10 ml YT medium containing ampicillin (50 μg/ml) overnight and transferred into 1 l YT/AMP medium. The cell cultures were incubated at 37°C to an optical density of 0.7 at 600 nm, and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. All samples were grown at 18°C overnight after induction except for those expressing AraGim1p which were grown at 37°C for 3 hours.

2.4.2 Cell lysate preparation

Fusion proteins produced from pGEX2T derivatives were prepared following the procedure outlined in Current Protocols in Protein Science (Harper and Speicher, 1997) with some modifications. Cells were harvested by centrifugation (5 000 x g for 15 minutes at 4°C) after overnight incubation, resuspended in 20 ml of resuspension buffer (50 mM Tris [pH 8.0], 2 mM EDTA, 0.15 mM PMSF, 1% Triton X-100, 2 mg of lysozyme) and mixed for 15 minutes at 30°C.

Cells carrying pTrcHis derivatives were harvested as recommended by the manufacturer (Clontech) with some modifications. The cells were harvested by centrifugation (5000 x g for 15 minutes at 4°C), resuspended in 16 ml of resuspension buffer (50 mM sodium phosphate [pH 7], 300 mM NaCl, 5 mM imidazole, 0.15 mM

PMSF, and 12 mg of lysozyme per g of *E. coli* cells) and mixed for 15 minutes at room temperature.

In both cases, the cells then were sonicated using the large tip of the Branson Sonifier 250. During sonication, samples were stored on ice and sonicated ten times using a 10 second pulse with a 20 second pause between each pulse. After centrifugation (40 000 x g for 30 minutes in the JA-20 rotor at 4°C), aliquots containing the same amount of protein from the supernatant or the cell debris pellet were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) as described in the following section to verify that the fusion protein had been produced. The supernatant containing the protein of interest was tested for protein concentration (see below) and used for column chromatography.

2.4.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gels consisting of a 7.5% or 10% or 12% or 15% separating gel and a 4% stacking gel were made according to the instructions accompanying the Bio-Rad apparatus and based on Laemmli (1970). To analyze the level of purity of the proteins of interest, fractions from different stages of purification were loaded and electrophoresed at constant voltage (200 V) for 45 minutes.

2.4.4 Coomassie blue staining

This Coomassie blue staining protocol was adapted from Wong *et al.* (2000). To visualize the proteins separated by SDS polyacrylamide gel electrophoresis, the gels were placed in a microwavable plastic box with 100 ml Fairbanks A staining solution (0.05% Coomassie blue R-250, 25% isopropanol, 10% acetic acid). The gels then were heated in a conventional 1000 W output microwave oven on full power for 2 minutes and cooled at room temperature for 5 minutes with gentle shaking. The Fairbanks A staining solution was discarded, distilled water at room temperature was added for rinsing, and immediately discarded. Approximately 50 ml of Fairbanks B staining solution (0.005% Coomassie, 10% isopropanol, 10% acetic acid) were added to the gels and the microwave procedure carried out for 1 minute 20 seconds. The hot Fairbanks B solution was discarded; room temperature distilled water was added and immediately discarded. The gels were transferred to 50 ml of Fairbanks C staining solution (0.002% Coomassie, 10% acetic acid) and the microwave procedure carried out again for 1 minute and 20 seconds. The gels were rinsed again with distilled water at room temperature and placed in about 50 ml Fairbanks D destaining solution (10% acetic acid) and placed again in the microwave for 1 minute and 20 seconds. A piece of Kimwipe was placed in the solution to absorb excess dye and the gels were allowed to cool at room temperature for 5

minutes with gentle shaking. Clear gel background could be achieved immediately if the Fairbanks D destaining step was repeated two or more times or if the gels were left shaking in the destaining buffer for about 15 minutes.

2.4.5 Protein concentration/bovine serum albumin standard curve

Protein concentrations were determined following the procedure supplied with the Bio-Rad protein assay kit. In brief, to 200 µl of dye reagent was added sample plus water to make a final volume of 1 ml. Absorbance was measured at 595 nm on a Perkin Elmer Cetus Lambda 3 spectrophotometer.

A standard curve was plotted with known amounts (0, 2.5, 5, 7.5, 10, 12.5 µg) of bovine serum albumin. Protein concentrations in different samples were determined by linear regression analysis from the standard curve.

2.4.6 Glutathione Sepharose 4B column chromatography

Purification using the Glutathione Sepharose 4B column followed the manufacturer's (Amersham Pharmacia Biotech) instructions using 5 ml of Glutathione Sepharose 4B gel in a chromatography column. The gel was packed in a 1 X 20 cm column by gravity to a bed volume of 5 cm. The column was washed with 5-10 bed

volumes of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, [pH 7.3]) to remove the preservative prior to each purification and equilibrated by 10 bed volumes of resuspension buffer (50 mM Tris [pH 8], 5mM EDTA and 1% Triton X-100). Cell lysate containing GST fusion protein was applied to the column and the column was washed with 200 bed volumes of PBS. The column was plugged and thrombin mix (10 µl thrombin [1U/1ul] in 1 ml of PBS) was added. The resin was left at 4 °C for about 24 hours with gentle shaking. After overnight incubation, the column was again packed by gravity and the flow through was collected. The protein of interest was in the eluate. The column was washed with 5 bed volumes of PBS, and any bound protein was eluted with 5 bed volumes of elution buffer (50 mM Tris [pH 8] and 20 mM glutathione). Fractions (1ml) were collected and analyzed by SDS-PAGE and for protein concentration.

The column was regenerated by washing with 2-3 bed volumes of alternating high pH (0.1 M Tris [pH 8.5] / 0.5 M NaCl) and low pH (0.1 M sodium acetate [pH 4.5] / 0.5 M NaCl) buffer. The cycle was repeated 3 times followed by re-equilibration with 3-5 bed volumes of 1X PBS. To remove precipitated or non-specifically bound proteins, the column was washed with 2 bed volumes of 6 M guanidine hydrochloride, followed by 5

bed volumes of 1X PBS and 3-4 bed volumes of 70% ethanol followed by a wash with 5 bed volumes of 1X PBS.

2.4.7 Metal affinity chromatography

His-tagged protein purification followed the manufacturer's (Clontech) instructions by batch-gravity elution. Talon CellThru Metal Affinity Resin (2 ml) was washed with 10 bed volumes of cold wash buffer (50 mM sodium phosphate [pH 7], 300 mM NaCl, 5 mM imidazole) and centrifuged at 700 x g for 5 minutes to pellet the resin. The supernatant was discarded and 10 bed volumes of cold wash buffer were added to resuspend the resin. The centrifugation was repeated and the supernatant discarded. The cell lysate then was added to the resin with mixing at 4°C for 30 minutes on a Labquake shaker. The mixture was transferred into a 2 ml CellThru disposable column (Clontech) and 20 bed volumes of wash buffer were added. Protein was eluted by gravity by adding 10 bed volumes of 50 mM sodium phosphate (pH 7), 300 mM NaCl, and 150 mM imidazole. Fractions (1 ml) were collected, analyzed by SDS-PAGE and tested for protein concentration. The resin was regenerated by washing with 5 bed volumes of 20 mM MES buffer (pH 5) / 0.1 M NaCl to remove imidazole, followed by 5 bed volumes of distilled water. The resin was stored in 20% ethanol at 4°C.

2.4.8 Co-elution from metal affinity column

Co-elution was carried out by following the metal affinity column protocol described in section 2.4.7 with some modifications. The Talon CellThru Metal Affinity Resin (2 ml) was washed with 10 bed volumes of cold wash buffer and centrifuged at 700 x g for 5 minutes to pellet the resin. The resin wash was repeated twice with wash buffer. Cell lysate containing His-tagged AraGim1p or AraYml079wp then was added to the resin with mixing at 4°C for 30 minutes on a shaker. The mixture was centrifuged at 700 x g for 5 minutes to pellet the resin. The resin was washed twice with wash buffer as described previously. The *Arabidopsis* or lupin tRNA nucleotidyltransferase in PBS was added to the resin and mixed at 4°C for 30 minutes on the Labquake shaker. The mixture then was transferred into a 2 ml CellThru disposable column (Clontech), 20 bed volumes of wash buffer were added to the column and the flow through was collected for SDS-PAGE analysis. Subsequently, the bound proteins were eluted as described in section 2.4.7.

2.4.9 Co-elution from Glutathione Sepharose 4B column

Purification using the Glutathione Sepharose 4B column followed the manufacturer's (Amersham Pharmacia Biotech) instructions using 5 ml of Glutathione

Sepharose 4B gel in a chromatography column. The gel was packed in a 1 X 20 cm column by gravity to a bed volume of 5 cm. The column was washed with 5-10 bed volumes of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, [pH 7.3]) to remove the preservative prior to each purification and equilibrated by 10 bed volumes of resuspension buffer (50 mM Tris [pH 8], 5 mM EDTA and 1% Triton X-100). Cell lysate containing GST fusion protein was applied to the column and 200 bed volumes of PBS were added. His-tagged AraGim1p or AraYml079wp in PBS was added to the resin and mixed at 4°C for 30 minutes on the Labquake shaker. The column was again packed by gravity and the flow through was collected. The column was washed with 5 bed volumes of PBS, and any bound protein was eluted with 5 bed volumes of elution buffer. Fractions (1ml) were collected, analyzed by SDS-PAGE and for protein concentration.

2.4.10 Measurement of tRNA nucleotidyltransferase activity

The standard procedure of Cudny *et al.* (1978) was used with some modifications. Each reaction mix contained 0.1 M glycine [pH 9.0], 10 mM $MgCl_2$, 0.2 mM CTP, 0.2 mM ATP, 11.2 µg yeast tRNA (Boehringer Mannheim) without CCA sequence (kindly supplied by Reesa Knight), 0.0216 µM [α^{32}P] ATP and 0.05 µg enzyme. This mixture

was incubated at room temperature in a glass tube for 20 minutes and the reaction stopped by adding 100 µl of cold 2 N HCl. Mixtures were left on ice for 20 minutes to precipitate the RNA and filtered through GF/C (Whatmann) glass fiber filters using a Millipore sampling manifold. Each filter then was washed with 100 ml of 1N HCl to eliminate any unincorporated free [α^{32}P] ATP. A final wash with 20 ml of 99% ethanol was done to facilitate drying of the filters. Filters were placed in the fume hood until they were completely dry and then placed in scintillation vials containing 5 ml scintillation fluid (Cytoscint from ICN). Counts were measured as cpm in triplicate in a scintillation counter (LKB Wallac-1218 Rack Beta).

2.4.11 Immunoblot

Protein was resolved on a 12% SDS-PAGE gel as described previously and electrophoretically transferred in a Trans-Blot SD semi-Dry Electrophoretic Transfer Cell (Bio-Rad) onto a nitrocellulose membrane in 39 mM glycine, 48 mM Tris, 20% methanol, and 0.037% SDS at 15V for 22 minutes (modified from manufacturer's specifications). Membranes were blocked 1 hour in 20 ml of 5% skim milk in TBS (20 mM Tris [pH 7.5], 0.5 M NaCl). Before adding the antibody, the membrane was washed three times with 20 ml TTBS (20 mM Tris [pH 7.5], 0.5 M NaCl, 0.05% Tween 20) for 15 minutes. The

following procedure was modified from the Western blotting procedure of Mai Wanru (personal communication). The membrane was treated with 10 ml of rabbit anti-tRNA nucleotidyltransferase antibody prepared by Fouad Karam (1:200 dilution in TTBS) or rabbit anti-GST antibody (Santa Cruz Biotechnology, 1:1000 dilution in TTBS) or rabbit anti-His-Tag polyclonal antibody (Cell Signaling Technology, 1:1000 dilution in TTBS) for 1 hour. The membrane then was washed twice with 20 ml TTBS for 5 minutes and then washed in 15 ml TTBS containing alkaline phosphatase-conjugated anti-Rabbit IgG secondary antibody (Promega) 1:7500 dilution for 1 hour. The membrane was washed twice with 20 ml TTBS and once with 20 ml TBS and color development was carried and in 10 ml of reaction buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5mM $MgCl_2$, 0.66% NBT, 0.33% BCIP). When a signal appeared on the membrane, water was added to stop the reaction. The membrane was blotted dry and stored in the dark.

2.4.12 Far Western blot

This protocol was adapted from Hall (2004). The first protein (lupin or *Arabidopsis* tRNA nucleotidyltransferase, or the AraGim1p or AraYml079wp) was subjected to electrophoresis, transfer and blocking as described in the immunoblot procedure. The second protein (AraGim1p or AraYml079wp if the first protein is tRNA

nucleotidyltransferase, or lupin tRNA nucleotidyltransferase if the first protein is AraGim1p or AraYml079wp) was added into blocking solution (5% skim milk in TBS) to a concentration of 4 μg/ml. The probe was incubated at room temperature for 2 hours. Before adding the antibody, the membrane was washed two times with 20 ml TTBS for 15 minutes. The membrane was treated with rabbit anti-tRNA nucleotidyltransferase antibody (1:200) (if the second protein is lupin tRNA nucleotidyltransferase) or with rabbit anti-His-Tag polyclonal antibody (1:1000) (if the second protein is a His-tagged protein) for 2 hours at room temperature and incubated at 4°C overnight. The membrane was washed twice with 20 ml TTBS for 5 minutes and then washed with 15 ml TTBS containing alkaline phosphatase-conjugated anti-Rabbit IgG secondary antibody (Promega) 1:7500 dilution for 1 hour. The membrane was washed twice with 20 ml TTBS and once with 20 ml TBS and color development was carried and in a 10 ml reaction buffer as described previously. When a signal appeared on the membrane, water was added to stop the reaction. The membrane was blotted dry and stored in the dark

2.4.13 Cross-linking

2.4.13.1 Glutaraldehyde

This protocol was adopted from Burns and Schachman (1982). Potentially interacting pairs of proteins (1-3 μg) were added in 50 μl aliquots in aqueous solution to Eppendorf tubes. This was followed by the addition of 12 μl of 0.25 M glutaraldehyde (grade 1, 25% aqueous solution diluted 1:10). This mixture was left at room temperature for 1 minute, followed by the addition of 20 μl of 1M $NaBH_4$ to stop the reaction. The 1M $NaBH_4$ was prepared just prior to addition to the reaction by diluting 0.38 mg in 1 ml of 0.1M NaOH. Samples were separated on a 12% polyacrylamide gel for analysis.

2.4.13.2 EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide ester)

This protocol was adopted from Grabarek and Gergely (1990). The first protein (1-3 μg) was mixed with 50 mM of EDC and 50 mM NHS. The reaction was left at room temperature for 15 minutes. After this incubation, 1 μl of 14 M β-mercaptoethanol was added to inhibit excess EDC. The second protein (1-3 μg) then

was added to the reaction tube in 50 µl and the mixture left at room temperature for one hour. Samples were separated on a 12% polyacrylamide gel for analysis.

2.5. Characterization of the yeast *YML079w* deletion

2.5.1 Test for cold and temperature sensitivity in different media

Cells were patched onto YPD medium (Table 2) and left at 30°C for 2 days. They then were replica plated to YPD medium (with or without 2.5 µg/ml of benomyl) and YPG medium and incubated at 14°C (7 days), 23°C (3 days), 30°C (overnight) or 37°C (overnight).

2.5.2 Tests for viability and respiratory competence

Cells were streaked on YPD plates (Table 2) and single colonies were picked for growth in 5 ml of YPD medium at 30°C with shaking at 250 rpm in an incubator-shaker. At each time point, an aliquot of 1 ml was removed and the OD_{600} determined. Cells were harvested by centrifugation at 4000 x g for 5 minutes at room temperature. The resulting cell pellet was diluted to 3 X 10^3 cells/ml (dilute 2 X 10^4 times from original) in 100 mM potassium phosphate (pH 7.4). Of this, 100 µl was spread on a YPD plate which was incubated at 30°C for 2 days. The colonies were counted and replica-plated

to YPD or YPG medium. The index of respiratory competence (IRC) was calculated as the number of colonies on the YPG plate divided by the number of colonies on the YPD plate multiplied by 100% (Shanmugam *et al.*, 1996). The percent viability was defined as the number of cells collected on day X that form colonies on the YPD plate divided by the number of cells collected on day one that form colonies on the YPD plate multiplied by 100.

2.5.3 Hydrogen peroxide challenges

Hydrogen peroxide (Caledon 30% w/v) was used to prepare H_2O_2 solutions. Yeast cells were treated in a similar manner to that described by Collinson and Dawes (1992). In brief, cells from YPG plates were inoculated into 100 ml YPD in an incubator-shaker at 30°C to an OD_{600} of 0.15. The samples were collected by centrifugation and washed with 100 mM potassium phosphate buffer (pH 7.4) and diluted 3 times in 100 mM potassium phosphate buffer (pH 7.4). Samples (5 ml) then were exposed to various concentrations of H_2O_2 for 1 hour at 30°C with shaking (225 rpm). The H_2O_2 treated cell culture then was collected by centrifugation (4000 x g for 5 minutes at room temperature) and resuspended in the 100 mM potassium phosphate buffer to a cell density of 1 X 10^4 cells/ml (100 times dilution). Aliquots (100 µl) of diluted cell

culture were spread on YPD plates and placed at 30°C. After 48 hours, colonies were counted as an indication of the number of viable cells. At this point the samples could be replica-plated to YPG to check respiratory competence.

3. Results

3.1. Plasmid construction

The open reading frames from ATG3 of the *Arabidopsis* and ATG2 of the lupin tRNA nucleotidyltransferases were PCR amplified from the vectors pzL1*Ara*CCA and Lupin 1D1, respectively, and fragments corresponding to the expected size of the open reading frames (1626 bp and 1509 bp) were generated (Figure 12).

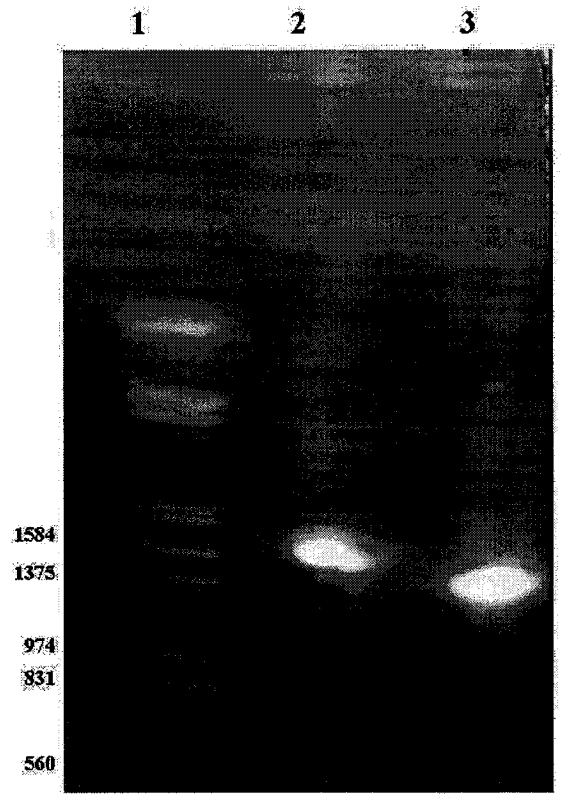


Figure 12: Agarose gel showing products generated from PCR amplification of the *Arabidopsis* and lupin tRNA nucleotidyltransferase open reading frames.

Lane 1 *Eco*RI-*Hin*dIII lambda marker, lane 2 PCR product of the *Arabidopsis* open reading frame starting from 3rd ATG, lane 3 PCR product of the lupin open reading frame starting from 2nd ATG.

The fragments were digested with the appropriate restriction enzymes and cloned into pGEX2T to generate pGEX2TaraCCA3 and pGEX2TlupinCCA2. Restriction digestion confirmed single insertion events and the correct orientation of the inserts (data not shown). Production of recombinant proteins was confirmed either by activity assay or Western blot.

3.2. Protein-protein interactions

Six recombinant proteins (*Arabidopsis* tRNA nucleotidyltransferase gene product starting from the first ATG, *Arabidopsis* tRNA nucleotidyltransferase starting from the third ATG, lupin tRNA nucleotidyltransferase starting from the first ATG, lupin tRNA nucleotidyltransferase starting from the second ATG, AraGim1p and AraYml079wp) were used for the protein-protein interaction experiments. The expression vectors used, the predicted sizes of the protein products and the abbreviations used to describe them are listed in Table 5.

3.2.1 Recombinant protein expression and purification

The *Arabidopsis* and lupin tRNA nucleotidyltransferases, the AraGim1p, and AraYml079wp were purified from *E. coli* extracts by affinity chromatography.

Table 5: Recombinant proteins used in the experiments.

Proteins	**Abbreviation**	**Expression vector**	**Size with tag (KDa)**	**Size without tag (KDa)**
***Arabidopsis* tRNA nucleotidyltransferase gene product starting from the first ATG**	Ara1 or A1	pGEX2T	94	67
***Arabidopsis* tRNA nucleotidyltransferase starting from the third ATG**	Ara3 or A3	pGEX2T	87	60
Lupin tRNA nucleotidyltransferase starting from the first ATG	Lup1 or L1	pGEX2T	90	62
Lupin tRNA nucleotidyltransferase starting from the second ATG	Lup2 or L2	pGEX2T	84	55
***Arabidopsis* homologue of the yeast *GIM1* gene product**	AraGim1p	pTrcHis	18	14
***Arabidopsis* homologue of the yeast *YML079w* gene product**	AraYml079wp	pTrcHis	25	21

3.2.1.1 GST-fusion proteins

After sonication, the fusion proteins were located primarily in the pellets (Figure 13). The predicted sizes of the GST-fusion proteins of the *Arabidopsis* (Ara1 and Ara3)

and lupin (Lup1 and Lup2) tRNA nucleotidyltransferases are 94 KDa (Ara1), 87 KDa (Ara3), 90 KDa (Lup1), and 84 KDa (Lup2), in good agreement with what was seen on the gels (Figure 13).

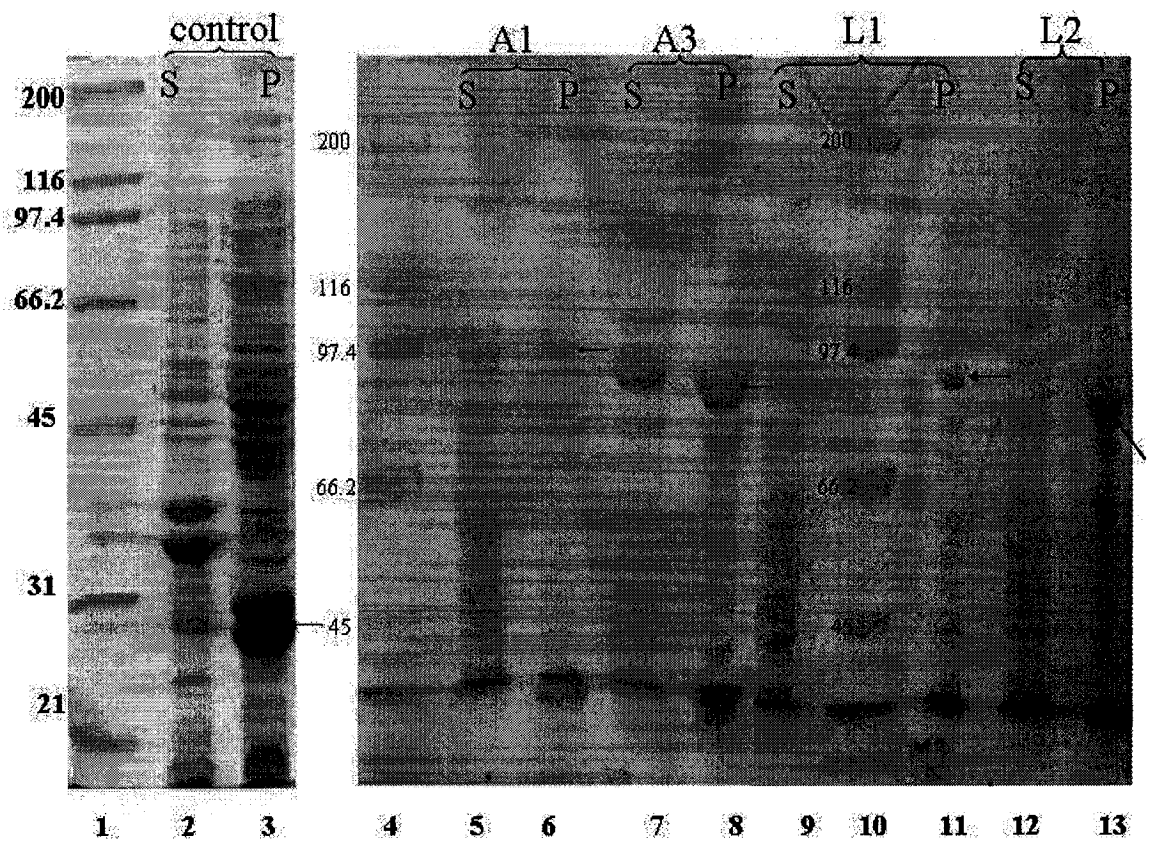


Figure 13: Over-expression of glutathione S-transferase (GST) fusion proteins.

Arrows indicate the predicted proteins. Lanes 1, 4 and 10 Bio-Rad SDS-PAGE molecular weight standards (broad range), lane 2 pellet of no insert control, lane 3 supernatant of no insert control, lane 5 supernatant of Ara1, lane 6 pellet of Ara1, lane 7 supernatant of Ara3, lane 8 pellet of Ara3, lane 9 supernatant of Lup1, lane 11 pellet of Lup1, lane 12 supernatant of Lup2, and lane 13 pellet of Lup2.

3.2.1.2 Cleavage of GST fusion protein

After the GST fusion proteins were cleaved by thrombin, only a small amount of pure tRNA nucleotidyltransferase was obtained except in the case of Ara3 (Figures 14 and 15). The purified proteins after thrombin cleavage have molecular weights of 67 KDa (Ara1), 60 KDa (Ara3), 62 KDa (Lup1), and 55 KDa (Lup2) as predicted (Figures 14 and 15).

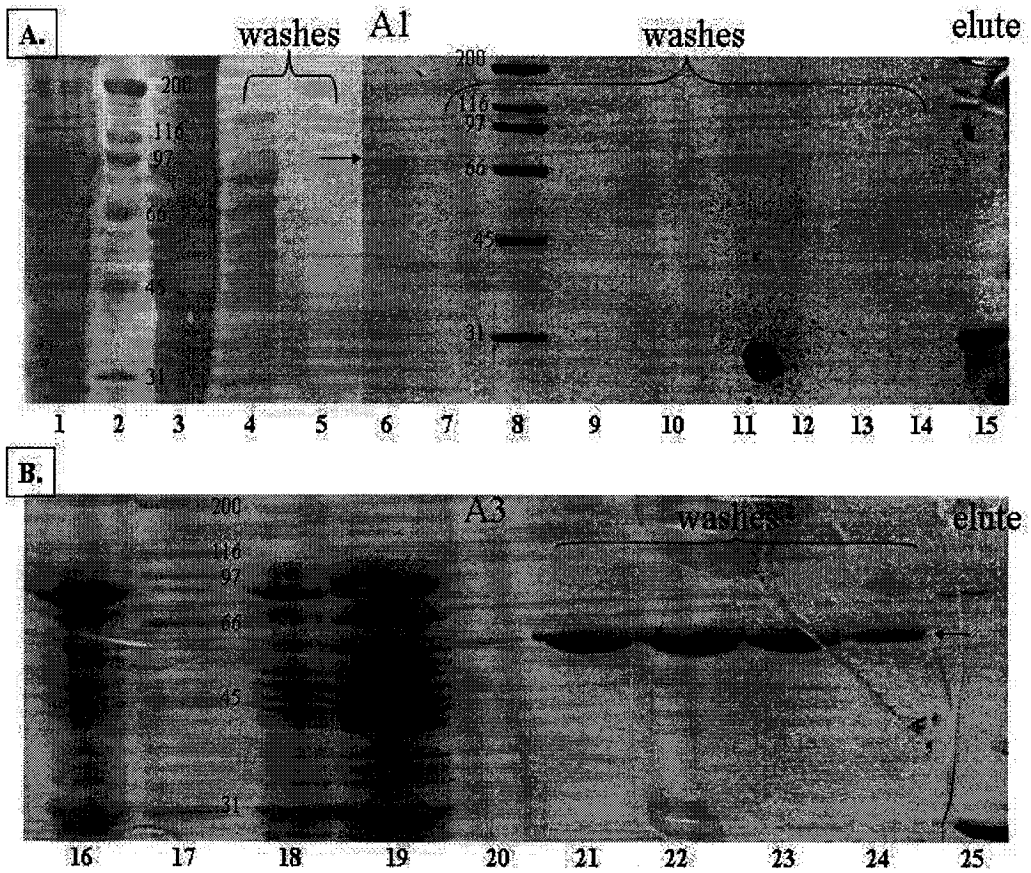


Figure 14: *Arabidopsis* tRNA nucleotidyltransferase purification using Glutathione Sepharose 4B resin.

Arrows indicate the predicted proteins. Panel A Ara1, panel B Ara3. Lanes 1 and 16 cell lysates before loading on column, lanes 2, 8 and 17 Bio-Rad SDS-PAGE molecular weight standards (broad range), lanes 3 and 19 cell lysate flow through from column,

lanes 4, 5, 7, 9-14, 18 and 21-24 washes with PBS, lane 6 Ara1 eluted after thrombin cleavage, lanes 15 and 25 bound proteins eluted with glutathione, lane 21 Ara3 eluted after thrombin cleavage.

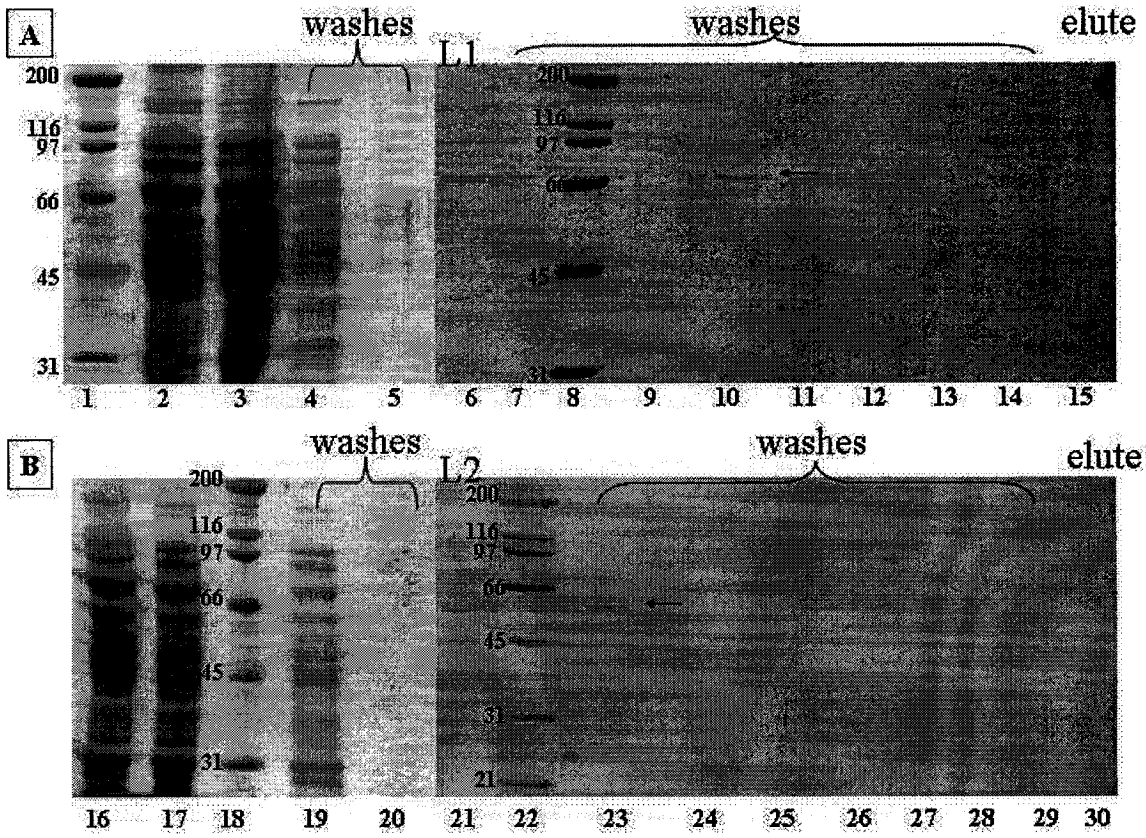


Figure 15: Lupin tRNA nucleotidyltransferase purification using Glutathione Sepharose 4B resin.

Arrows indicate the predicted proteins. Panel A Lup1, Panel B Lup2. Lanes 1, 8, 18 and 22 Bio-Rad SDS-PAGE molecular weight standards (broad range), lanes 2 and 16 cell lysates before loading on column, lanes 3 and 17 cell lysate flow through from column, lanes 4, 5, 7, 9-14, 19, 20, 23-29 washes with PBS, lane 6 Lup1 eluted after thrombin cleavage, lanes 15 and 30 bound proteins eluted with glutathione, lane 21 Lup2 eluted after thrombin cleavage.

3.2.1.3 Assays for tRNA nucleotidyltransferase activity

Pure *Arabidopsis* and lupin tRNA nucleotidyltransferases with and without GST tags were used in enzyme activity assays to check if the enzymes produced in *E. coli* were functional. Only Ara3GST and Ara3 were active. The other tRNA nucleotidyltransferases were inactive (Figure 16).

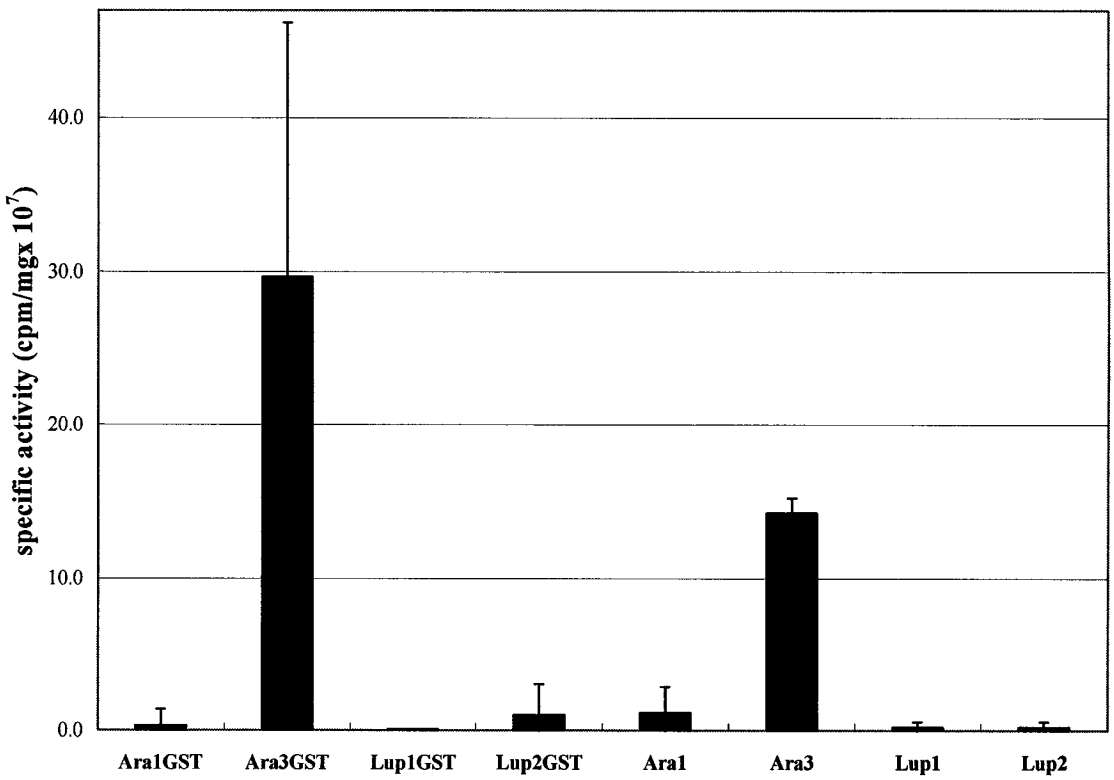


Figure 16: Activity of *Arabidopsis* and lupin tRNA nucleotidyltransferases generated in *E. coli*. Error bars indicate standard deviation from the mean.

3.2.1.4 His-tagged fusion proteins

The His-tagged fusion proteins corresponding to AraGim1p and AraYml079wp are seen at 18 KDa and 25 KDa as predicted (Figure 17).

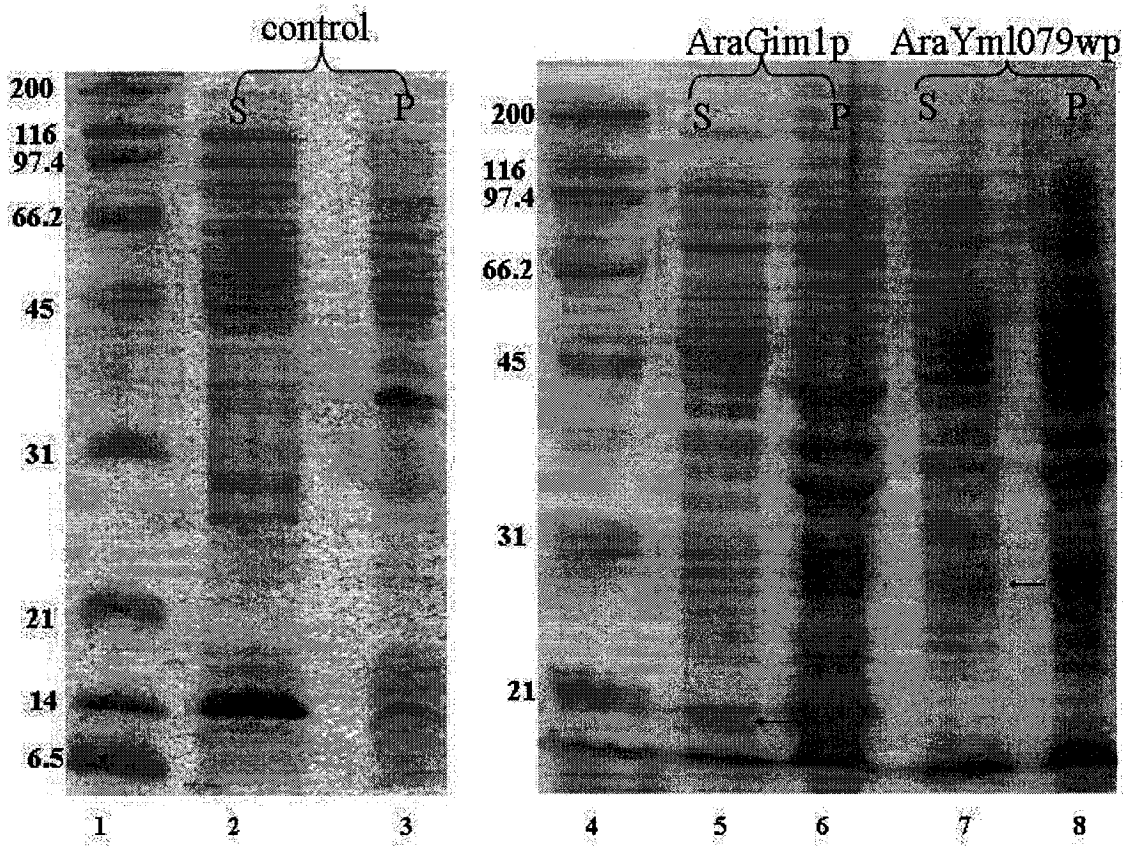


Figure 17: Over-expression of polyhistidine-tagged fusion proteins.

Arrows indicate the predicted fusion proteins. Lanes 1 and 4 Bio-Rad SDS-PAGE molecular weight standards (broad range), lane 2 supernatant of no insert control, lane 3 pellet of no insert control, lane 5 supernatant of AraGim1p, lane 6 pellet of AraGim1p, lane 7 supernatant of AraYml079wp, and lane 8 pellet of AraYml079wp.

His-tagged fusion proteins were purified through a cobalt affinity column. The purified fusion proteins of AraGim1p and AraYml079wp have molecular weights of 18 KDa and 25 KDa respectively (Figure 18).

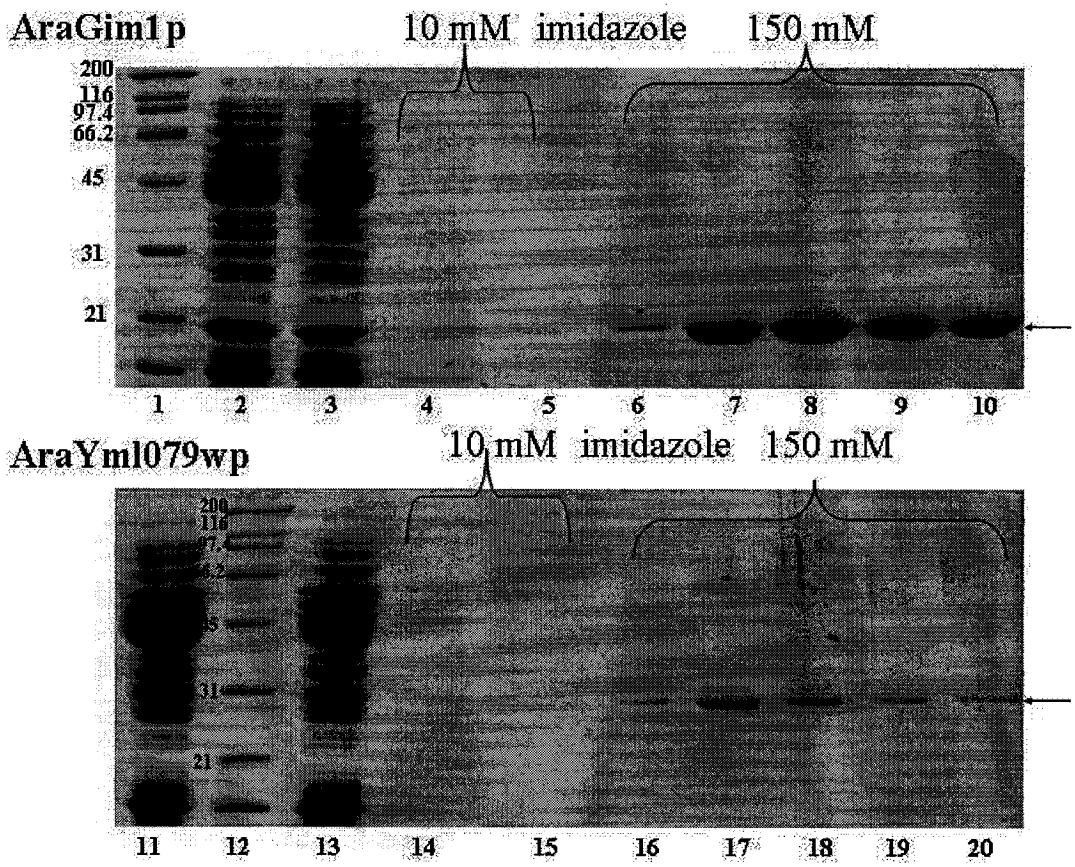


Figure 18: AraGim1p and AraYml079wp purified by metal affinity chromatography

Lane 1 and 12 Bio-Rad SDS-PAGE molecular weight standards (broad range), lane 2 cell lysate before loading on column, lane 3 and 13 cell lysate flow through from column, lanes 4, 5, 14 and 15 washes with 10 mM imidazole, lanes 6-10 AraGim1p eluted with 150 mM imidazole, lanes 16-20 AraYml079wp eluted with 150 mM imidazole.

3.2.2 Far-Western blot

When His-tagged AraGim1p and AraYml079wp were bound to the membrane, the lupin tRNA nucleotidyltransferase containing its mitochondrial/chloroplast targeting signal showed an interaction with AraYml079wp (Figure 19, panel B). In contrast, no interaction was observed in the case of the lupin tRNA nucleotidyltransferase without the organelle targeting signal (Figure 19, panel C).

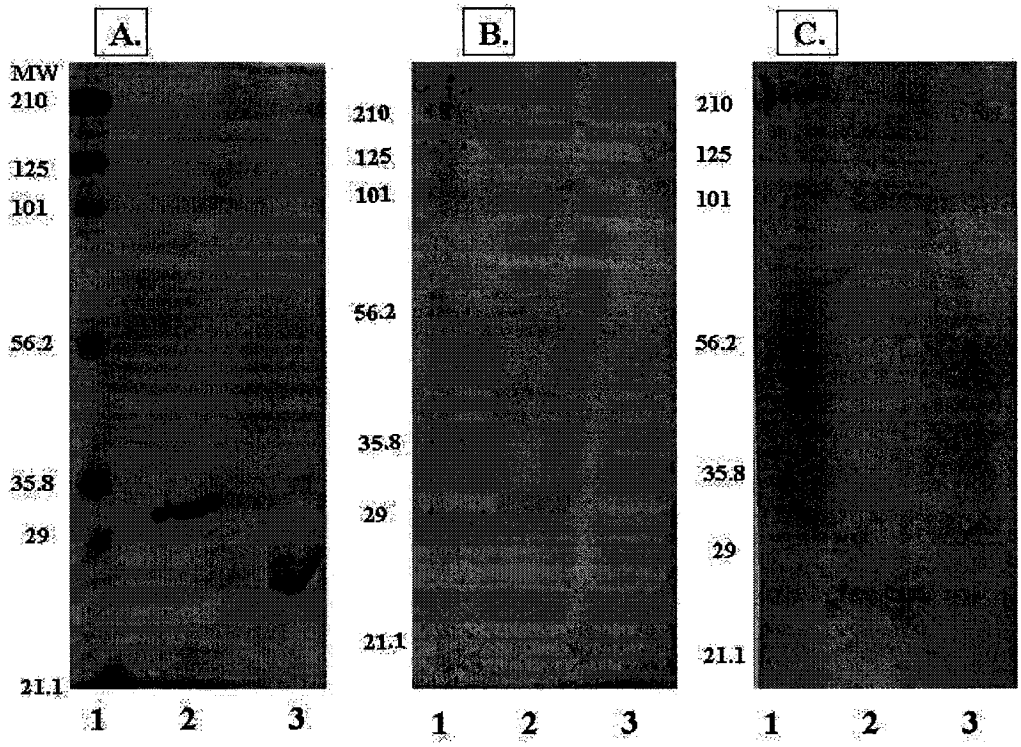


Figure 19: Far Western blot using the lupin tRNA nucleotidyltransferase as the probe protein.

Lane 1 Bio-Rad prestained SDS-PAGE standards (broad range), lane 2 AraYml079wp and lane 3 AraGim1p. A) SDS-PAGE before transfer, B) far Western blot using Lup1 (containing targeting signal) as probe protein using Lupin antibody for the detection, C) far Western blot using Lup2 (lacking the targeting signal) as probe protein using Lupin antibody for the detection.

When the reciprocal experiment was performed using AraGim1p or AraYml079wp as probe protein, only the *Arabidopsis* tRNA nucleotidyltransferase with its organellar targeting signal showed an interaction with either probe protein (Figure 20). The interaction between lupin tRNA nucleotidyltransferase and the AraYml079wp did not show in this case (Figure 20).

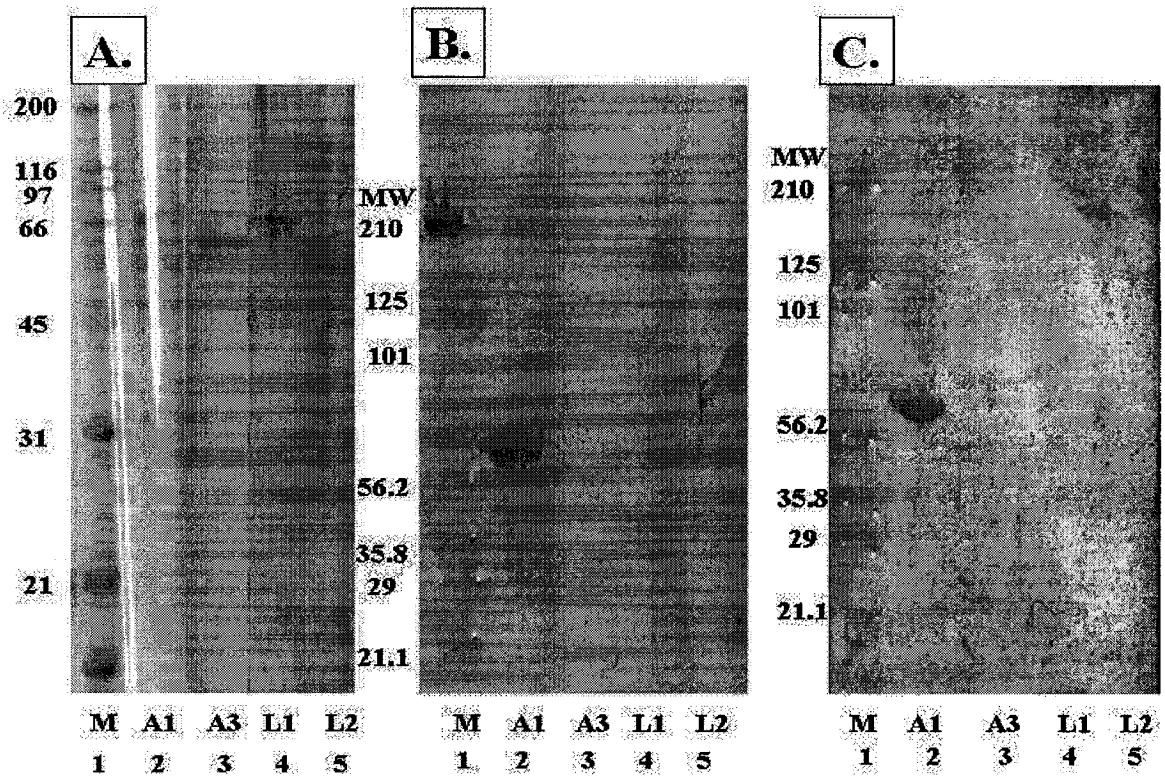


Figure 20: Far Western blot using the polyhistidine-tagged fusion proteins as the probe proteins.

Lane 1 Bio-Rad prestained SDS-PAGE standards (broad range), lane 2 Ara1, lane 3 Ara3, lane 4 Lup1 and lane 5 Lup2. A) SDS-PAGE, equal amount of the proteins were loaded as for far Western blot, B) far Western blot using AraYml079wp as probe protein using His-tag antibody for the detection , C) far Western blot using AraGim1p as probe protein using His-tag antibody for the detection.

3.2.3 Co-elution

Using the bound histidine-tagged AraGim1p or the bound histidine-tagged AraYml079wp as probe proteins, the lupin and *Arabidopsis* tRNA nucleotidyltransferases were used to demonstrate an interaction by co-elution. Only the lupin tRNA nucleotidyltransferase with its organelle targeting signal (Lup1) showed an interaction with AraYml079wp (Figure 21). The lupin tRNA nucleotidyltransferase without the extra 57 amino-terminal amino acids did not show any interaction with AraYml079wp (Figure 21). When AraGim1p was used as bait protein, the lupin tRNA nucleotidyltransferase did not show any interaction (Figure 22). No interaction occurred between the *Arabidopsis* tRNA nucleotidyltransferase with or without organelle targeting signal and any bound histidine-tagged protein (Figures 23 and 24).

3.2.4 Cross-linking

Two cross-linkers were used to test for an interaction between tRNA nucleotidyltransferase and the His-tagged proteins (AraGim1p or AraYml079wp). When glutaraldehyde was used as the cross-linker, the *Arabidopsis* or lupin tRNA nucleotidyltransferases showed self-interaction. The high molecular weight species also were present when the His-tagged proteins were added (Figure 25).

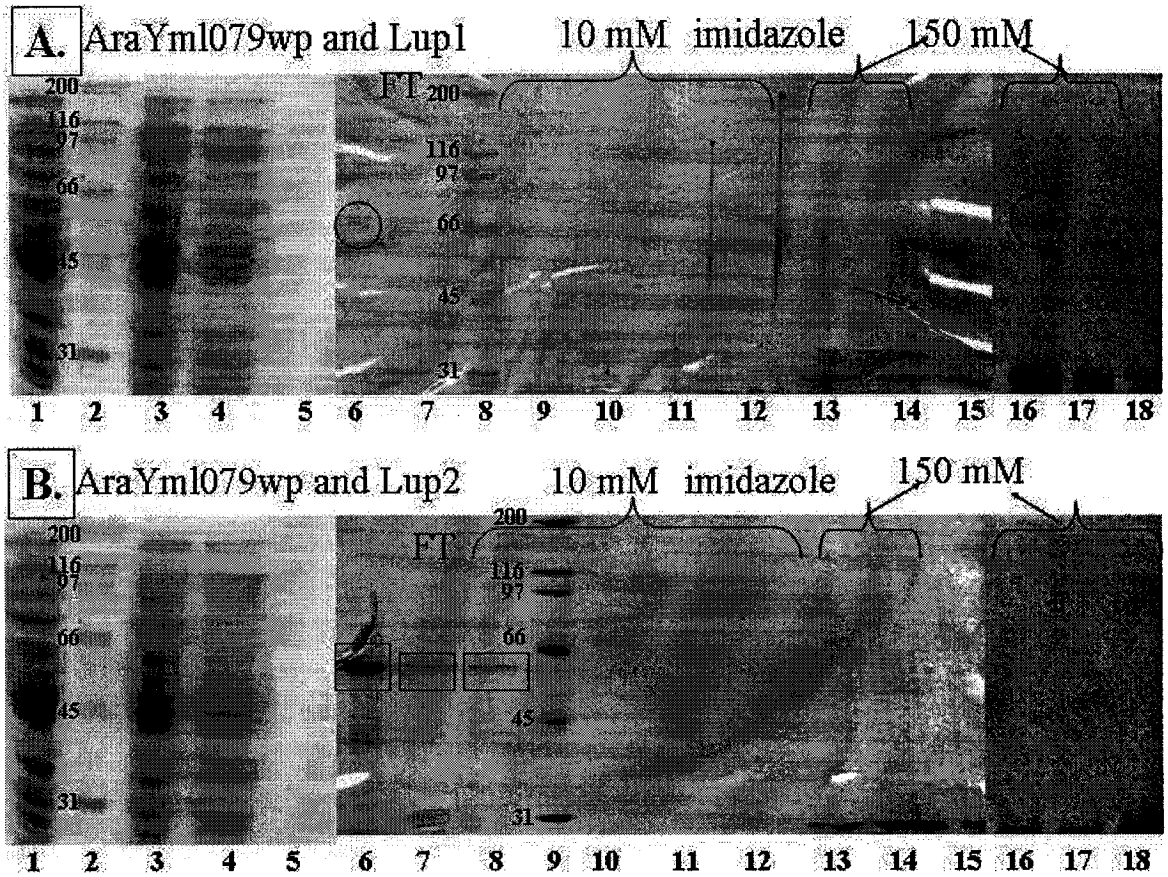


Figure 21: Co-elution using AraYml079wp as the bait protein and the lupin tRNA nucleotidyltransferase with (L1) or without (L2) the organellar targeting signal as the prey protein.

Panel A: lane 1 AraYml079wp cell lysate before applying to column, lanes 2 and 8 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 3 AraYml079wp cell lysate flow through from the column, lanes 4, 5, 9-12 washes with 10mM imidazole, lane 6 L1 before mix, lane 7 L1 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraYml079wp. L1 protein bands are circled.

Panel B: lane 1 AraYml079w p cell lysate before applying to column, lanes 2 and 9 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 3 AraYml079w p cell lysate flow through from the column, lanes 4, 5, 8 and 10-12 washes with 10mM imidazole, lane 6 L2 before mix, lane 7 L2 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane 15 AraYml079wp. L2 protein bands are boxed.

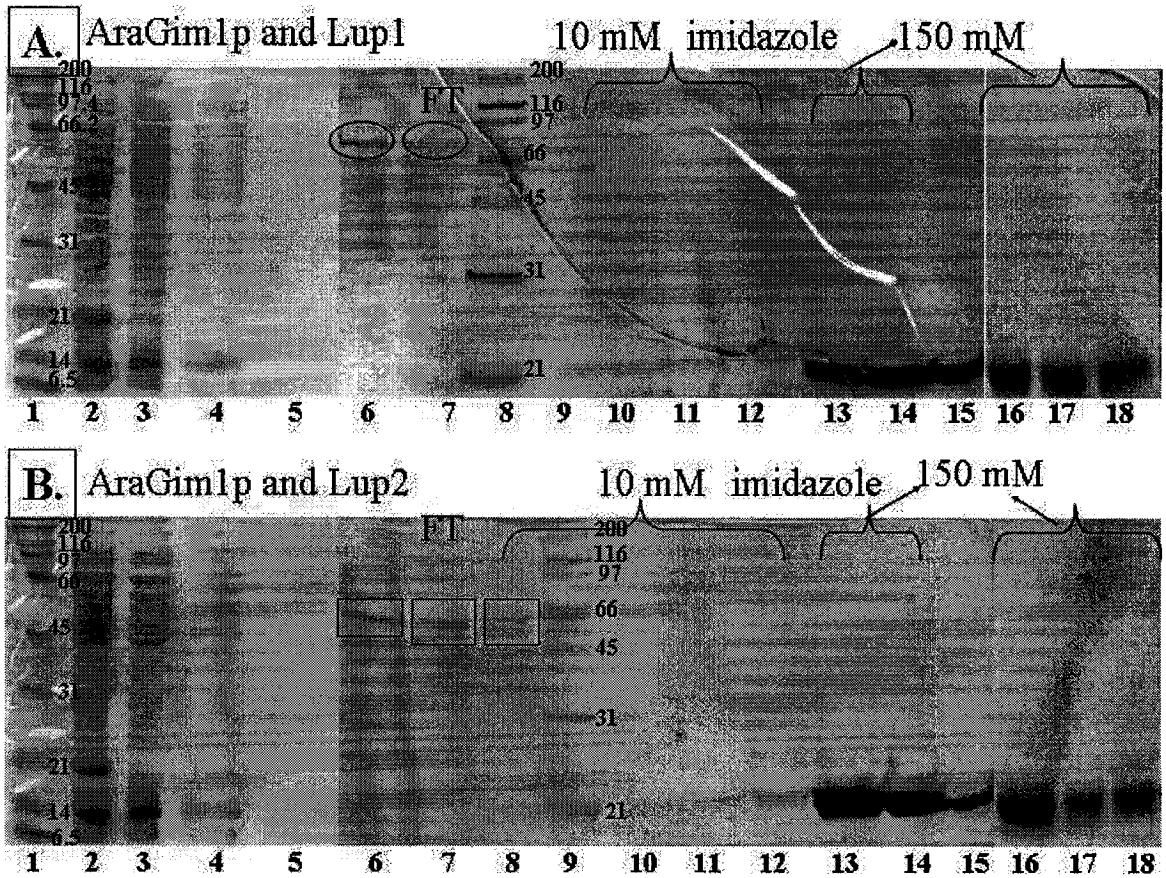


Figure 22: Co-elution using AraGim1p as the bait proteins and the lupin tRNA nucleotidyltransferase with (L1) or without (L2) the organellar targeting signal as the prey protein.

Panel A: lanes 1 and 8 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 2 AraGim1p cell lysate before applying to column, lane 3 AraGim1p cell lysate flow through from the column, lanes 4, 5, and 9-12 washes with 10mM imidazole, lane 6 L1 before mix, lane 7 L1 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150mM imidazole., lane 15 AraGim1p. L1 protein bands are circled.

Panel B: lanes 1 and 9 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 2 AraGim1p cell lysate before applying to column, lane 3 AraGim1p cell lysate flow through from the column, lanes 4, 5, 8 and 10-12 washes with 10mM imidazole, lane 6 L2 before mix, lane 7 L2 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraGim1p. L2 protein bands are boxed.

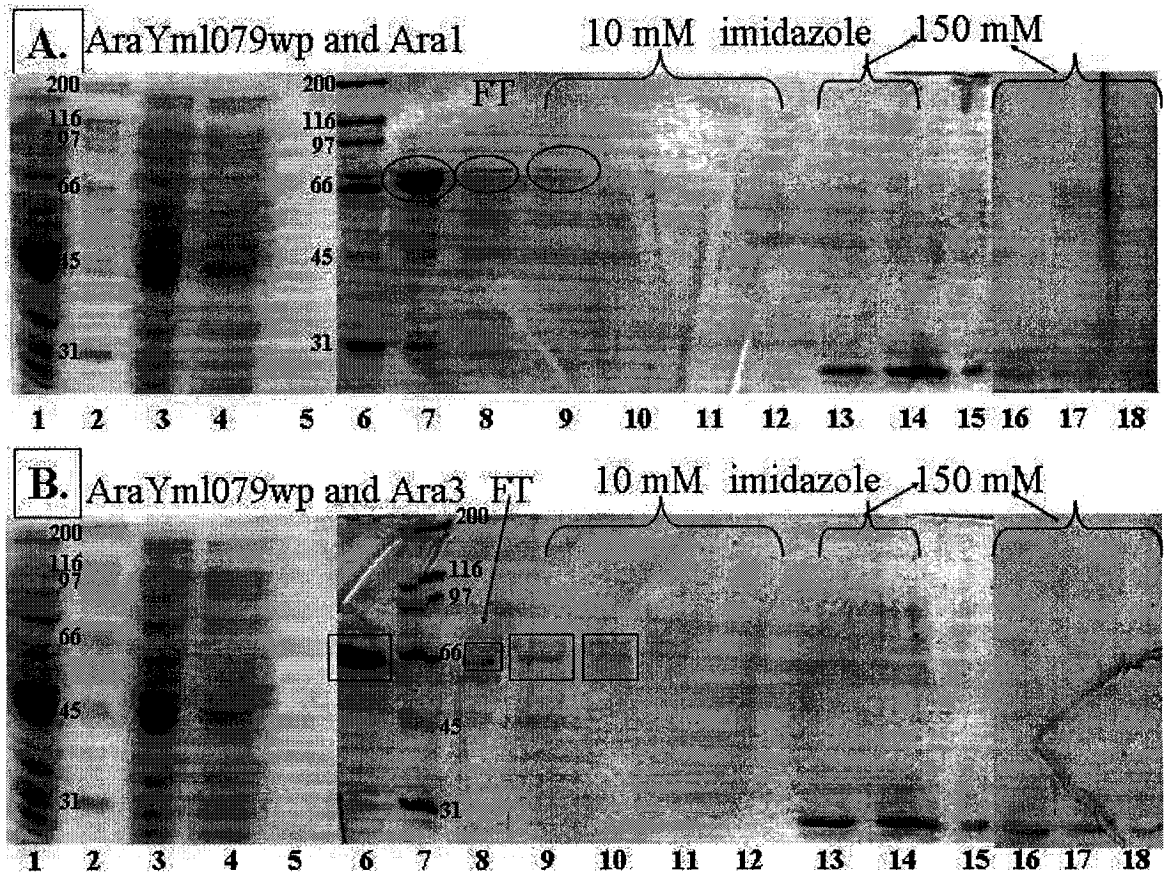


Figure 23: Co-elution using AraYml079wp as the bait proteins and the *Arabidopsis* tRNA nucleotidyltransferase with (A1) or without (A3) the organellar targeting signal as the prey protein.

Panel A: lane 1 AraYml079wp cell lysate before applying to column, lanes 2 and 6 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 3 AraYml079wp cell lysate flow through from the column, lanes 4, 5, and 9-12 washes with 10mM imidazole, lane 7 A1 before mix, lane 8 A1 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraYml079wp. A1 protein bands are circled.

Panel B: lane 1 AraYml079wp cell lysate before applying to column, lane 2 and 7 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 3 AraYml079wp cell lysate flow through from the column, lanes 4, 5, and 9-12 washes with 10mM imidazole, lane 6 A3 before mix, lane 8 A3 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraYml079wp. A3 protein bands are boxed.

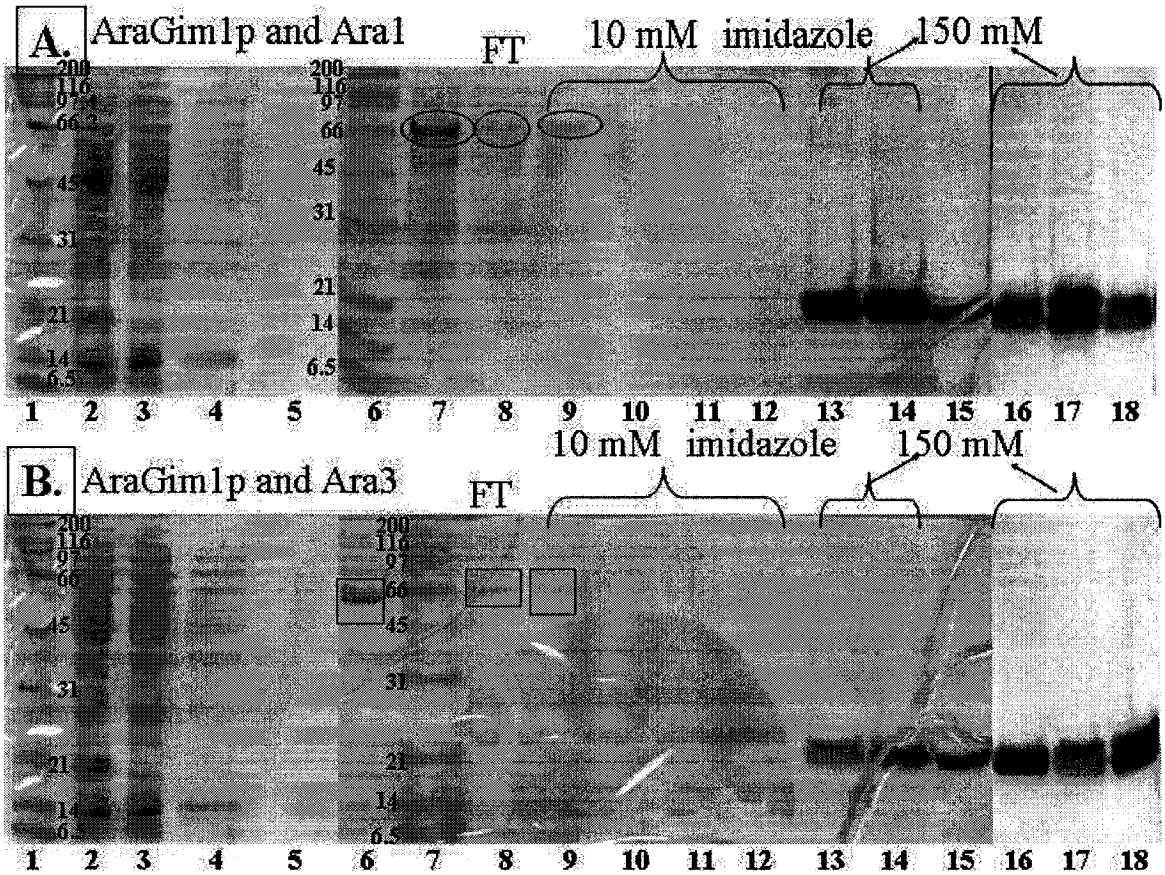


Figure 24: Co-elution using AraGim1p as the bait protein and the *Arabidopsis* tRNA nucleotidyltransferase with (A1) or without (A3) the organellar targeting signal as the prey protein.

Panel A: lanes 1 and 6 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 2 AraGim1p cell lysate before applying to column, lane 3 AraGim1p cell lysate flow through from the column, lanes 4, 5, and 9-12 washes with 10mM imidazole, lane 7 A1 before mix, lane 8 A1 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraGim1p. A1 protein bands are circled.

Panel B: lanes 1 and 7 Bio-Rad SDS- PAGE molecular weight standards (broad range), lane 2 AraGim1p cell lysate before applying to column, lane 3 AraGim1p cell lysate flow through from the column, lanes 4, 5, and 9-12 washes with 10mM imidazole, lane 6 A3 before mix, lane 8 A3 flow through from the column, lanes 13, 14 and 16-18 bound protein eluted from the column with 150 mM imidazole, lane15 AraGim1p. A3 protein bands are boxed.

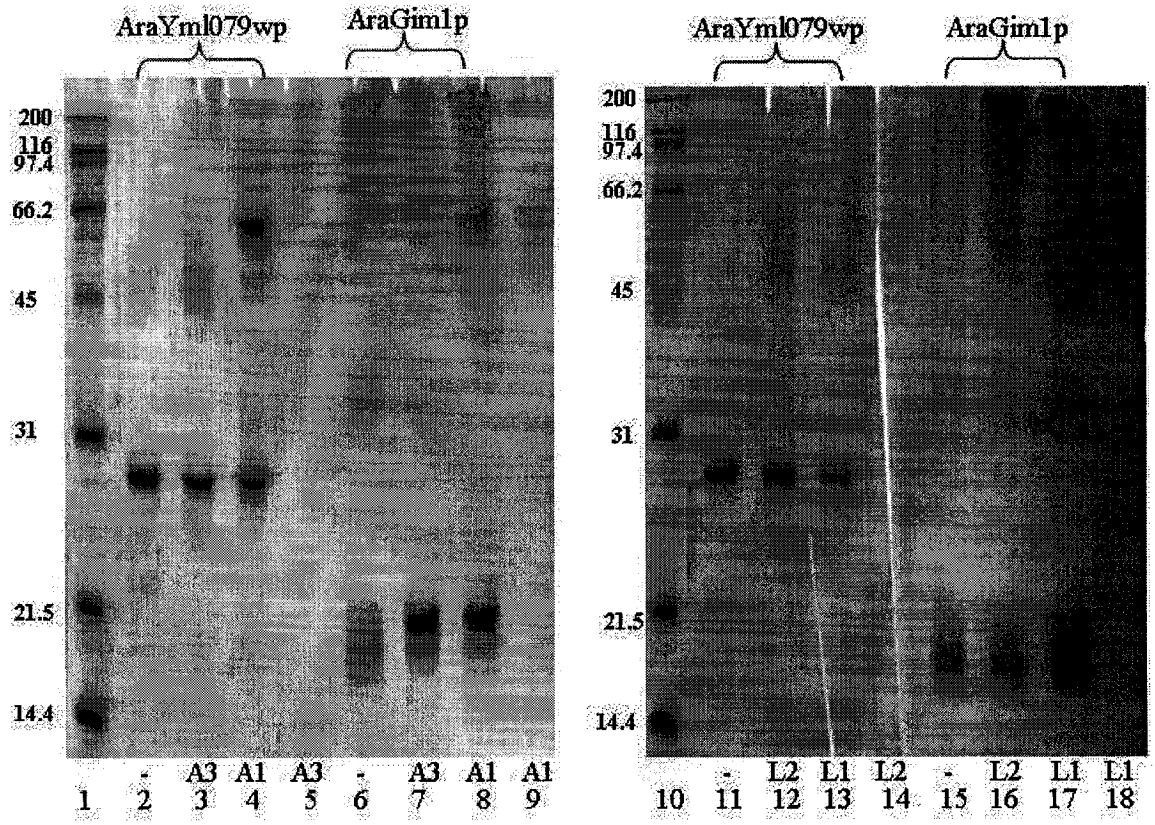


Figure 25: Cross-linking using glutaraldehyde as cross-linker.

Lanes 1 and 10 Bio-Rad SDS-PAGE molecular weight standards (broad range), lanes 2 and 11 AraYml079wp and cross-linker, lane 3 A3 cross-linked with AraYml079wp, lane 4 A1 cross-linked with AraYml079wp, lane 5 A3 and cross-linker, lanes 6 and 15 AraGim1p and cross-linker, lane 7 A3 cross-linked with AraGim1p, lane 8 A1 cross-linked with AraGim1p, lane 9 A1 and cross-linker, lane 12 L2 cross-linked with AraYml079wp, lane13 L1 cross-linked with AraYml079wp, lane 14 L2 and cross-linker, lane 16 L2 cross-linked with AraGim1p, lane 17 L1 cross-linked with AraGim1p, lane 18 L1 and cross-linker.

No interaction between *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p or AraYml079wp was apparent when EDC and NHS were used as cross-linkers (Figure 26). However, the lupin tRNA nucleotidyltransferase can interact with AraYml079wp. The

amount of the lupin tRNA nucleotidyltransferase was decreased when lupin tRNA nucleotidyltransferase was added first. The self association observed when glutaraldehyde was used as cross-linker did not show when EDC and NHS were used (Figure 26).

3.2.5 Summary of interaction results

The results of interactions shown in this study are summarized in tables 6 and 7 along with the two-hybrid data of Gu (2000). The two-hybrid data for an interaction between AraGim1p and the *Arabidopsis* tRNA nucleotidyltransferase with its targeting signal are supported by the far Western blot data using His-tagged AraGim1p as probe protein (Figure 20). In contrast, the data from co-elution (Figure 24) and cross-linking (Figures 25 and 26) are either inconclusive or do not support this interaction. According to the yeast two-hybrid data, lupin tRNA nucleotidyltransferase containing or lacking its targeting signal showed no interaction with AraGim1p. These results were supported by the far Western (Figure 20), co-elution results (Figure 22) and cross-linking data (Figures 25 and 26).

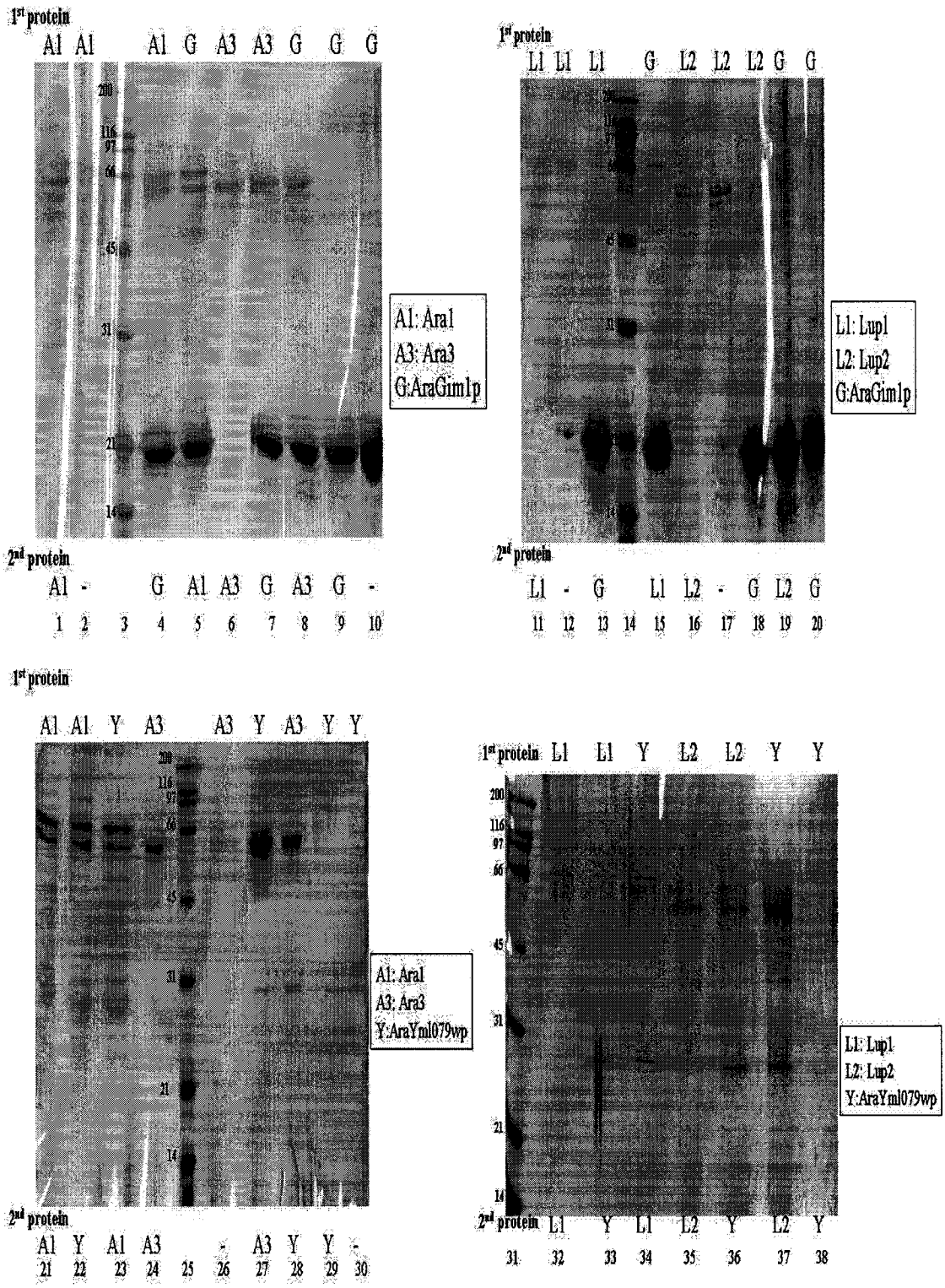


Figure 26: Cross-linking using EDC and NHS as cross-linkers.

Lanes 1 and 21 A1 cross-linked with A1, lane 2 A1 without cross-linker, lanes 3, 14, 25 and 31 Bio-Rad SDS-PAGE molecular weight standards (broad range), lane 4 A1 cross-linked with AraGim1p, lane 5 AraGim1p cross-linked with A1, lanes 6 and 24 A3 cross linked with A3, lane 7 A3 cross-linked with AraGim1p, lane 8 AraGim1p cross linked with A3, lanes 9 and 20 AraGim1p cross-linked with AraGim1p, lane 10 AraGim1p without cross-linker, lanes 11 and 32 L1 cross-linked with L1, lane 12 L1 without cross-linker, lane 13 L1 cross-linked with AraGim1p, lane 15 AraGim1p cross-linked with L1, lanes 16 and 35 L2 cross linked with L2, lane 17 L2 without cross-linker, lane 18 L2 cross-linked with AraGim1p lane 19 AraGim1p cross linked with L2, lane 22 A1 cross-linked with AraYml079wp, lane 23 AraYml079wp cross-linked with A1, lane 26 A3 without cross-linker, lane 27 AraYml079wp cross linked with A3, lane 28 A3 cross-linked with AraYml079wp, lane 29 and 38 AraYml079wp cross-linked with AraYml079wp, lane 30 AraYml079wp without cross-linker, lane 33 L1 cross-linked with AraYml079wp, lane 34 AraYml079wp cross-linked with L1, lane 36 L2 cross linked with AraYml079wp, lane 37 AraYml079wp cross-linked with L2.

From Table 7, both the co-elution (Figure 21) and far Western blot results (Figure 19) support the yeast two-hybrid data showing an interaction between the lupin tRNA nucleotidyltransferase (with its targeting signal) and AraYml079wp. However, when His-tagged AraYml079wp fusion protein was used as the probe protein in a far Western assay, the lupin tRNA nucleotidyltransferase did not show an interaction (Figure 20). From yeast two-hybrid data, *Arabidopsis* tRNA nucleotidyltransferase with or without its targeting signal did not interact with AraYml079wp. The far Western data (Figure 20) showed that when His-tagged AraYml079wp was used as a probe a clear positive signal

could be observed. Co-elution and cross-linking results were either inconclusive or supported the yeast two-hybrid data.

Table 6: Interactions between AraGim1p and tRNA nucleotidyltransferase

tRNA nucleotidyltransferase	***Arabidopsis***	***Arabidopsis***	**Lupin**	**Lupin**
Targeting signal	**Yes**	**No**	**Yes**	**No**
Yeast two-hybrid assay (Gu, 2000)	+	-	-	-
Far Western blot using His-tag protein as probe protein	+	-	-	-
Far Western blot using lupin tRNA nucleotidyltransferase as probe protein	**NA**	**NA**	-	-
Co-elution	-	-	-	-
Cross-linking using glutaraldehyde	**?**	**?**	**?**	**?**
Cross-linking using EDC and NHS	-	-	-	-

+: interaction, -: no interaction, NA: not applicable, ? uncertain interaction.

Table 7: Interactions between AraYml079wp and tRNA nucleotidyltransferase

tRNA nucleotidyltransferase	***Arabidopsis***	***Arabidopsis***	**Lupin**	**Lupin**
Targeting signal	**Yes**	**No**	**Yes**	**No**
Yeast two-hybrid assay (Gu, 2000)	-	-	+	-
Far Western blot using His-tag protein as probe protein	+	-	-	-
Far Western blot using lupin tRNA nucleotidyltransferase as probe protein	**NA**	**NA**	+	-
Co-elution	-	-	+	-
Cross-linking using glutaraldehyde	**?**	**?**	**?**	**?**
Cross-linking using EDC and NHS	-	-	+	+

+: interaction, -: no interaction, NA: not applicable, ? uncertain interaction.

3.3. Characterization of the yeast *YML079w* deletion

By searching GenBank, the yeast *YML079w* gene product was identified as a homologue of AraYml079wp, the product of the *Arabidopsis* Atlg19130 gene, found using tRNA nucleotidyltransferase as bait in the yeast two-hybrid system. The yeast *YML079w* deletion strain (Giaever *et al.*, 2002) was used to explore the possible function

of this unknown protein in yeast. The literature indicated that this was not an essential gene in yeast (Giaever *et al.*, 2002).

3.3.1 Cold and temperature sensitivity in different carbon sources

The *S. cerevisiae YML079w* deletion strain and an isogenic strain wild-type at the *YML079w* locus were tested for their ability to grow at different temperatures and utilizing different carbon sources. Compared to NT33-5 (a temperature sensitive strain), deletion of *YML079w* did not affect viability at 37°C (Figure 27). Also, no significant difference was seen between growth of strains BY4743ΔHO and BY4743ΔYML079w at low temperature (14°C) (Figure 27). Unlike SGY101 p415-ADH (which is a cold and benomyl sensitive strain), addition of benomyl did not affect the growth of BY4743ΔYML079w (Figure 27). On the non-fermentable carbon source at all different temperatures tested, NT33-5 (at 37°C) and SGY101 p415-ADH (at 37°C and 14°C) did not grow well. The rest of the yeast strains can grow on YPG (Figure 27). At 22°C and 30°C, all of the strains can grow on the fermentable carbon source with or without benomyl.

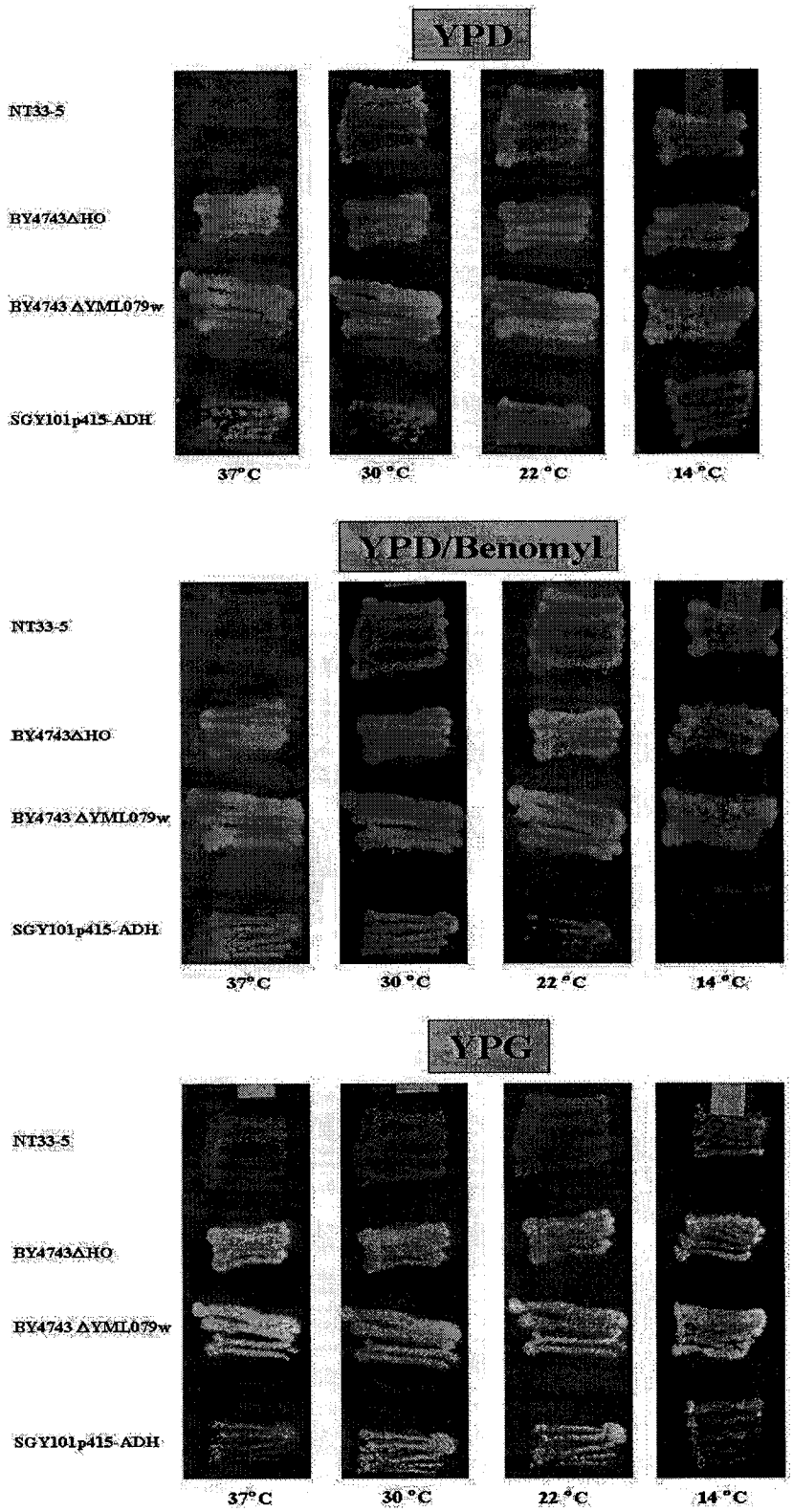


Figure 27: Ability of yeast strains BY4743ΔHO, BY4743ΔYML079w, NT33-5 and SGY101 p415-ADH to grow on different carbon sources and at different temperatures.

3.3.2 Growth curves

To determine if deletion of *YML079w* will affect doubling times or affect the ability of the cells to leave stationary phase, BY4743ΔYML079w and the isogenic wild-type strain were grown on a fermentable carbon source. Cells were grown overnight and diluted to an OD_{600} of 0.05 in 50 ml of fresh YPD medium and grown at 30°C. Under these conditions the growth curves for both strains are virtually indistinguishable (Figure 28).

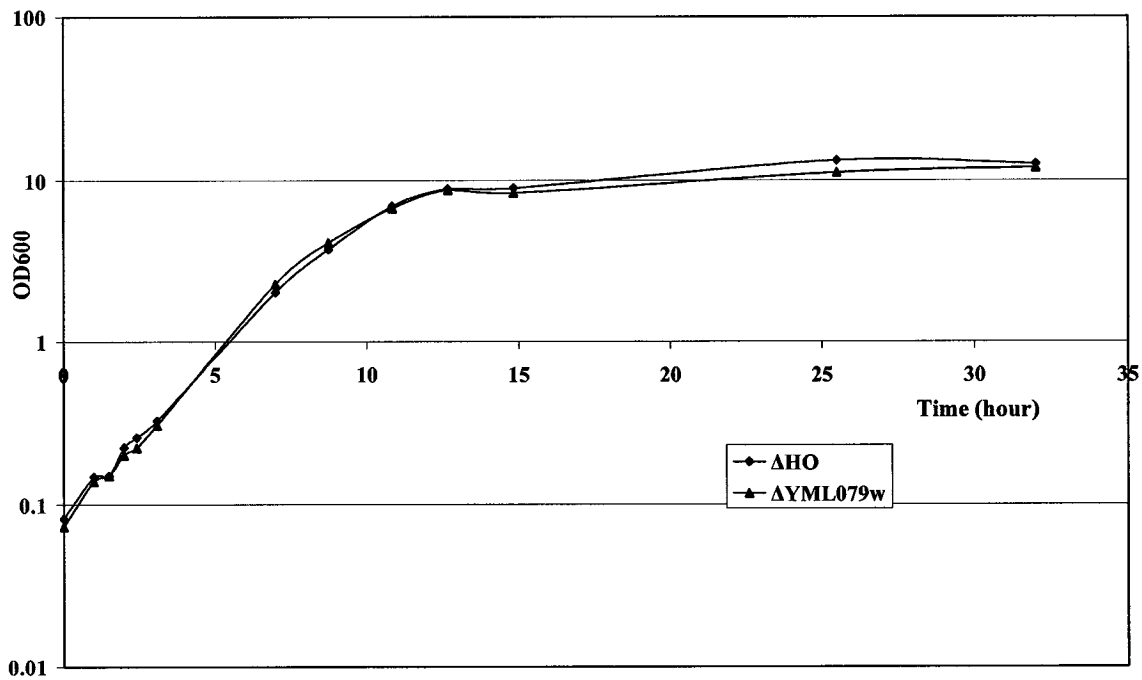


Figure 28: Comparison of the growth of the strains BY4743ΔHO and BY4743ΔYML079w, at 30°C in YPD with shaking.

3.3.3 Viability and respiratory competence

To determine whether the *YML079w* deletion had any effect on long term viability, BY4743ΔHO and BY4743ΔYML079w were grown overnight to stationary phase in YPD and diluted in 50 ml of fresh medium to an OD_{600} of 0.05. Incubation at 30°C was continued with shaking for 7 days and aliquots (1 ml) were taken at specific time points. At each time point, the samples were prepared as described in materials and methods, and incubated at 30°C for 2 days. There is no significant difference in long term viability between the wild type and *YML079w* deletion strains (Figure 29).

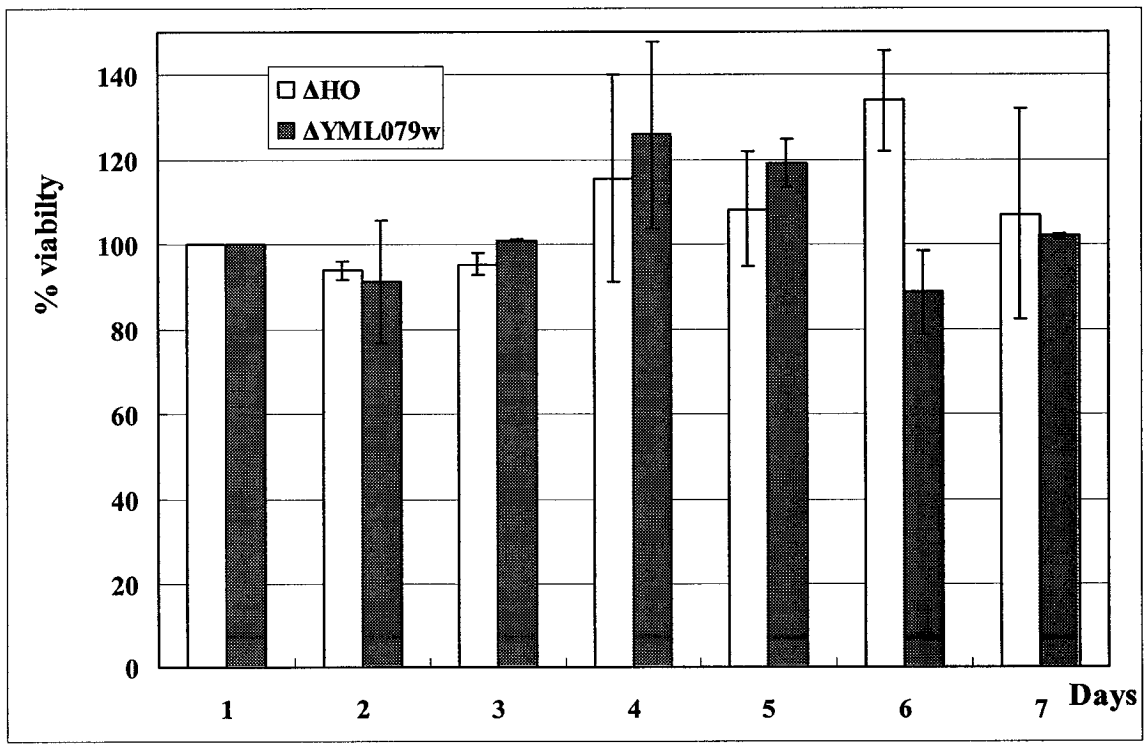


Figure 29: Comparison of viability of BY4743ΔHO and BY4743ΔYML079w. Error bars indicate standard deviation from the mean.

The colonies that grew on YPD medium were replica-plated to YPG medium at 30°C and respiratory competence was scored as the ability to grow on the non-fermentable carbon source. This also showed no difference between the two strains (Figure 30).

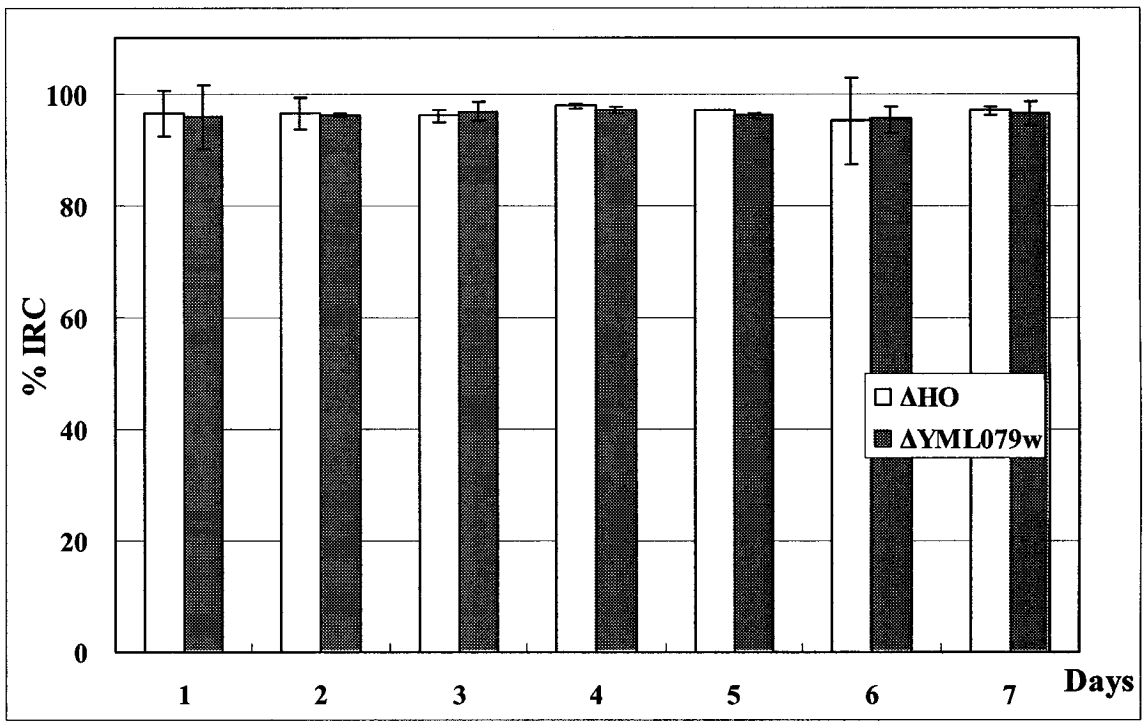


Figure 30: Comparison of index of respiratory competence (IRC) of BY4743ΔHO and BY4743ΔYML079w. Error bars indicate standard deviation from the mean.

3.3.4 Hydrogen peroxide challenge

To determine if *YML079w* had a role in protecting cells from H_2O_2-induced stress, cells were grown to an OD_{600} of 0.15 (early exponential phase) and challenged with various concentrations of H_2O_2 for a period of 1 hour. It can be seen that BY4743ΔYML079w

cells have a negligible difference in H_2O_2-induced cell death as compared to the isogenic wild-type cells and to BY4743ΔCCP1 (Figure 31). Moreover, when the colonies that grew on YPD medium were replica-plated to YPG medium at 30°C and respiratory competence was compared among the three strains, there is no difference between

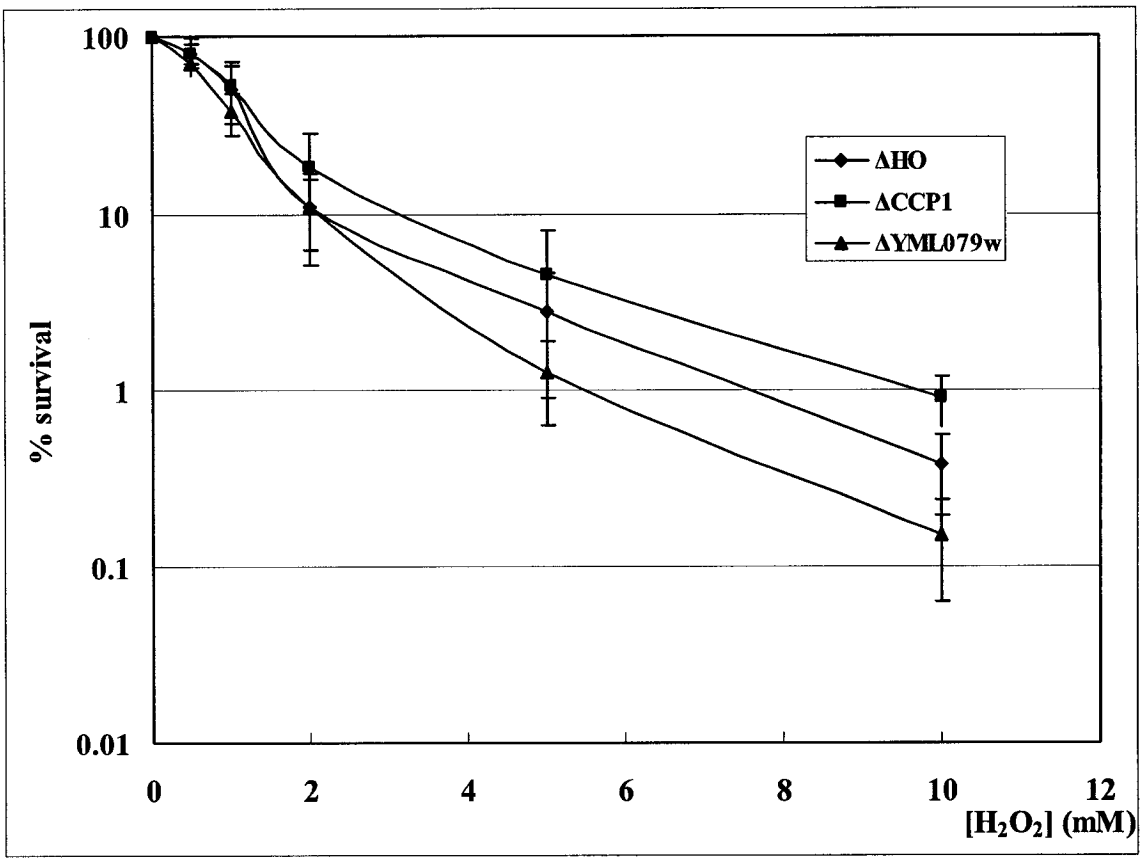


Figure 31: Susceptibility of exponential-phase BY4743ΔHO, BY4743ΔYML079w and BY4743ΔCCP1 *S. cerevisiae* strains to H_2O_2-induced stress. Error bars indicate standard deviation from the mean.

BY4743ΔYML079w and BY4743ΔHO, although BY4743ΔCCP1 showed about a 20% decrease of IRC compared to BY4743ΔHO at the 0.5 and 1 mM H_2O_2 (Figure 32). Since the survival rate at the 2, 5 and 10 mM H_2O_2 concentrations are low, the standard error is great, and no IRC was determined for these concentrations.

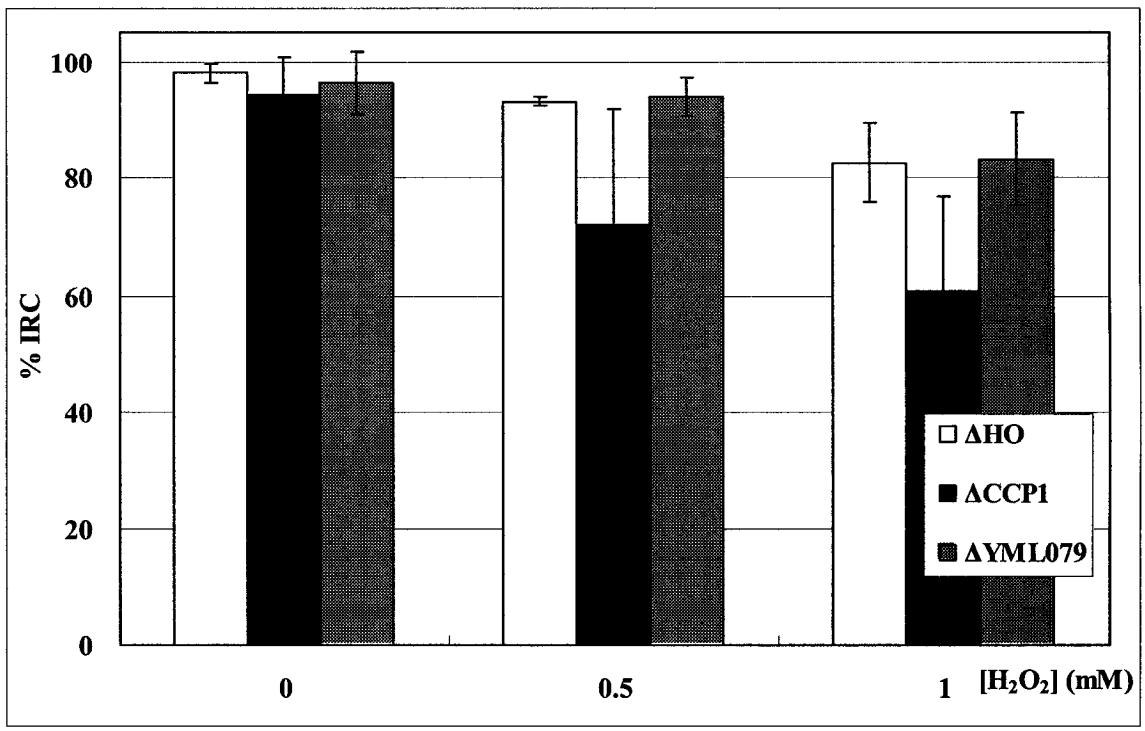


Figure 32: Index of respiratory competence (IRC) of BY4743ΔHO, BY4743ΔYML079w and BY4743ΔCCP1 after treatment with hydrogen peroxide. Error bars indicate standard deviation from the mean.

4. Discussion

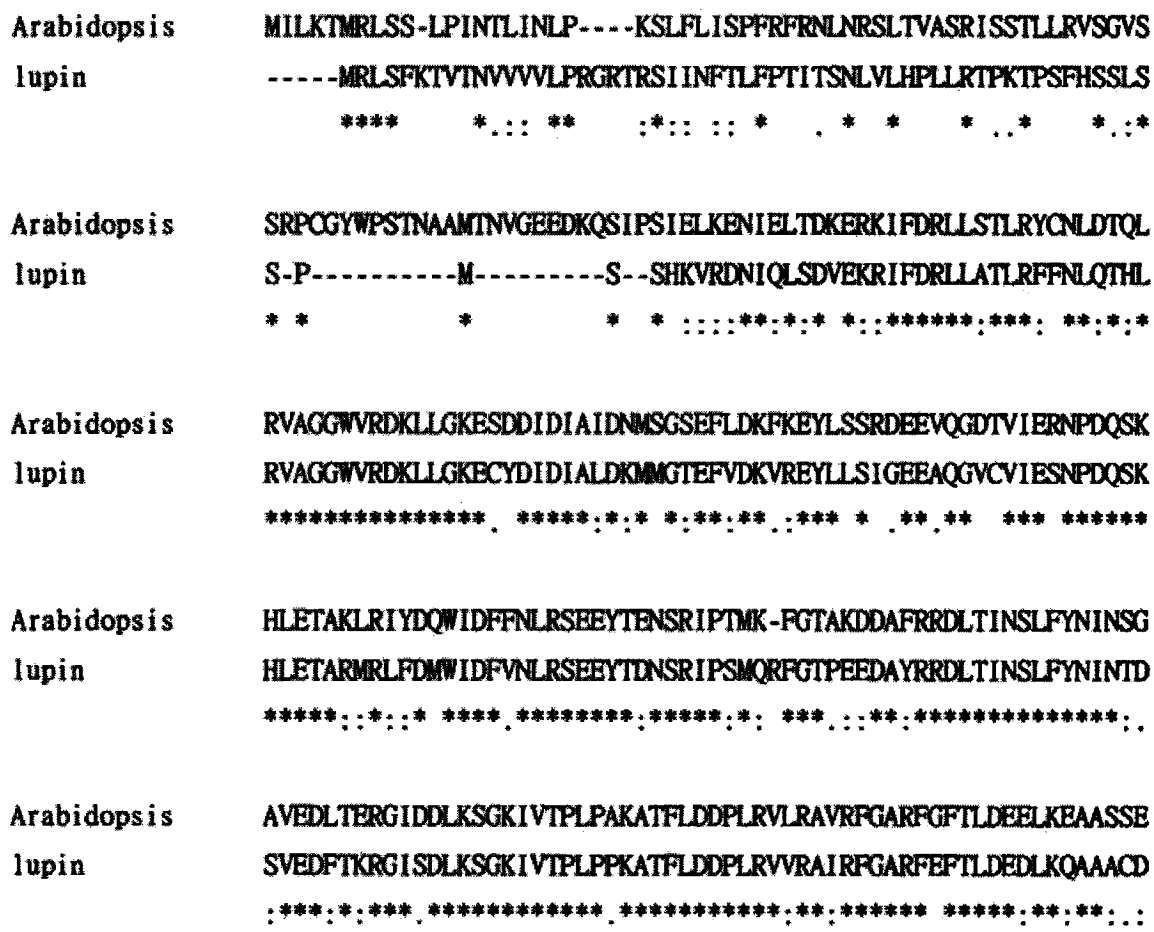
In a previous study, using the yeast two-hybrid system, results suggested that AraYml079wp interacted with lupin tRNA nucleotidyltransferase that contained its amino terminal targeting signal while AraGim1p interacted with *Arabidopsis* tRNA nucleotidyltransferase that contained its targeting signal. Both of these interactions were dependent on the presence of the targeting signals. The biological significance of these observations was brought into question by the subsequent observation that similar interactions were not seen in experiments using the yeast homologues of these proteins (http://biodata.mshri.on.ca/yeast_grid/servlet/SearchResults? keywords=YLR200W, http://biodata.mshri.on.ca/yeast_grid/servlet/SearchResults? keywords=YML079W). In the study presented here, several biochemical methods were used to attempt to confirm the interactions suggested by the yeast two-hybrid data between the plant proteins. In addition, an attempt was made to define the function of AraYml079wp.

4.1. Recombinant proteins

4.1.1 Expressing tRNA nucleotidyltransferase

To study protein-protein interactions, GST fusion proteins were generated with the *Arabidopsis* and lupin tRNA nucleotidyltransferase gene products both containing and

lacking their amino-terminal targeting signals. Previous experiments in our lab had shown that expressing these plant proteins directly in *E. coli* resulted in extremely low yields (unpublished observations). This is one reason that the proteins were expressed in the experiments described here as GST-tagged proteins. Although the lupin and *Arabidopsis* tRNA nucleotidyltransferases have 48% sequence identity (Figure 33), the recombinant proteins showed differing solubilities in *E. coli*.



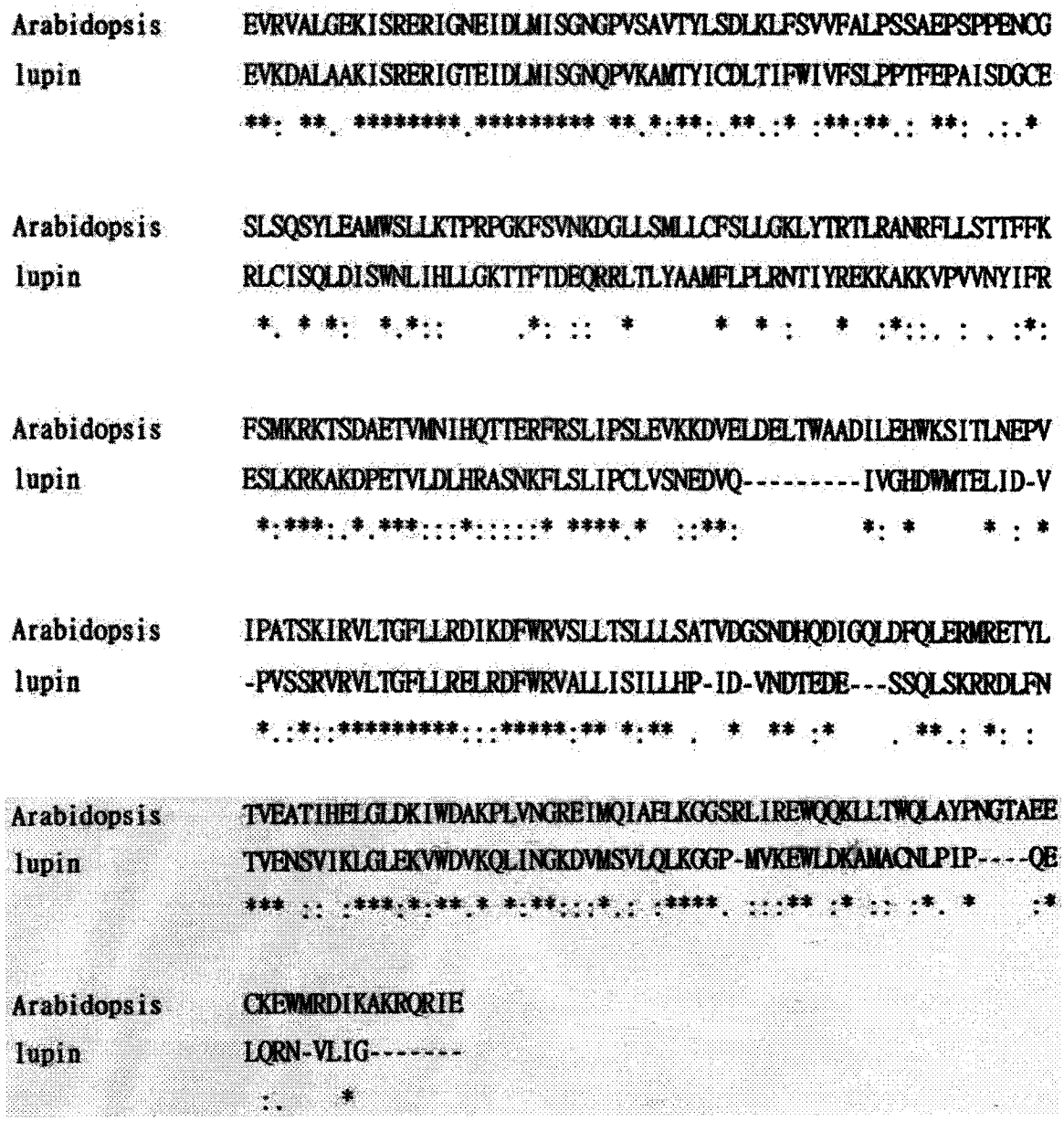


Figure 33: Amino acid sequence similarity of *Arabidopsis* and lupin tRNA nucleotidyltransferases.

Standard one letter abbreviations are used for the amino acids, identical amino acids in the two sequences are indicated "*", ":" indicates a strongly similar amino acid in the two sequences, "." indicated weakly similar amino acids and "-" indicates a gap introduced to optimize alignments.

According to the recombinant protein solubility prediction program of Wilkinson and Harrison (1991) (http://www.biotech.ou.edu), both the *Arabidopsis* and lupin tRNA nucleotidyltransferases without their amino terminal targeting signals are predicted to be 52% soluble when over-expressed in *E. coli*. The addition of the extra amino-terminal organellar targeting signal is predicted to shift this distribution to about 65% insoluble. This is in good agreement with what was seen experimentally for the *Arabidopsis* tRNA nucleotidyltransferase. The *Arabidopsis* protein is more soluble without its amino terminal targeting signal than with its amino terminal targeting signal (Figure 13). However, the result with the lupin tRNA nucleotidyltransferase did not agree with the predictions. The lupin tRNA nucleotidyltransferase with or without its amino terminal targeting signal was localized primarily in the insoluble fraction after sonication (Figure 13). The difference between the results seen with the *Arabidopsis* and lupin proteins might be because the presence of charged and turn forming residues, as used in the Wilkinson-Harrison statistical solubility model, is not the only critical factor that influences the solubility of the recombinant proteins in *E. coli*. Another factor that might influence the formation of soluble tRNA nucleotidyltransferase in *E. coli* is the hydrophobicity of these proteins. Although many of the hydrophobic groups in proteins are buried within the molecule to minimize their contact with water, some do contact

water, and these hydrophobic interactions affect protein solubility. The Kyte and Doolittle formula (Kyte and Doolittle, 1982) provides a way to calculate the average hydrophilicity of a protein, assigning negative values to charged amino acids like arginine and glutamate, and positive values to hydrophobic residues like methionine and isoleucine. The average hydrophilicity (AH) of a protein is calculated as the sum of the individual hydrophathy indices (HI) multiplied by the number of corresponding residues (n) divided by the total residues of the proteins [AH = (Σ HI*n)/Σn]. This calculation showed that the *Arabidopsis* tRNA nucleotidyltransferase (-0.32 for Ara1, -0.39 for Ara3) was more hydrophilic than the lupin protein (-0.15 for Lup1 and -0.19 for Lup2). This is in good agreement with what was seen experimentally (Figure 13). Other factors such as cysteine content of the proteins also may be involved. Since the *E. coli* cytoplasmic environment is reducing, it is most probable that disulfide bond formation occurs upon air contact after lysis (Mitraki and Jonathan, 1989). The disulfide bonds might cause the incorrect folding or aggregation of the protein on lysis. Because more cysteine residues are found in the lupin tRNA nucleotidyltransferase (8 cysteines in both Lup1 and Lup2) than in the *Arabidopsis* protein (5 cysteines in Ara1, 4 in Ara3), it may be these cysteine residues that influence the solubility of the protein.

As the yield of soluble protein in either case was sufficient for further experiments, the insoluble fractions were discarded and only the soluble fractions were used. This avoided the refolding steps that would have been required to isolate additional protein from the insoluble fractions. To characterize the tRNA nucleotidyltransferase proteins the GST portion of the fusion protein had to be removed by thrombin cleavage. However, the thrombin cleavage while the fusion protein was immobilized on the affinity matrix was very ineffective such that after the GST-tag was removed by thrombin cleavage only a small amount of pure tRNA nucleotidyltransferase was obtained except in the case of the *Arabidopsis* tRNA nucleotidyltransferase lacking its targeting signal (Figures 15 and 16). In some cases when cleavage on the column was not efficient, the fusion proteins were eluted from the column, cleaved in solution and then applied back to the glutathione column. Unfortunately, the cleaved GST tag did not bind well to the column and it was difficult to separate the cleaved GST tag from the tRNA nucleotidyltransferase, because GST exists as a dimer which has about the same molecular weight as tRNA nucleotidyltransferase. Additional attempts to separate the GST tag from tRNA nucleotidyltransferase (*e.g.*, by using ammonium sulfate precipitation) were unsuccessful. Also, the activity of the tRNA nucleotidyltransferase was lost after elution, cleavage and elution from a second column. Therefore, to obtain

purest protein, the GST tag was cleaved when the protein was still bound to the glutathione column even though the amount of tRNA nucleotidyltransferase obtained was small.

To avoid other ions that may interfere with enzyme activity (Shanmugam *et al.*, 1996), all assays were carried out in PBS. Since after the purification scheme the concentration of each tRNA nucleotidyltransferase was different, the concentration of each protein was adjusted with PBS to 0.01 μg/μl before activity assays. Even after purification under these conditions only the purified *Arabidopsis* tRNA nucleotidyltransferase lacking its amino terminal targeting signal was active (Figure 17).

Both the *Arabidopsis* and lupin tRNA nucleotidyltransferases with their mitochondrial/chloroplast targeting signals were unable to produce active protein. This might be because the protein with the organellar targeting signal does not fold correctly. Under normal conditions, the protein containing its amino-terminal targeting signal would be directed to the mitochondrion or chloroplast where the targeting signal would be removed as a part of the import process (Jarvis and Soll, 2001, Stojanovski *et al.*, 2003). Perhaps the targeting signal functions to keep the protein from folding into its native conformation so that it can be imported more efficiently into the mitochondrion or chloroplast (Miller *et al.*, 1991). There are numerous examples of recombinant proteins

that are soluble, but which have not folded into their native conformations and exist instead as soluble folding intermediates that are not biologically active (Sachdev and Chirgwin, 1998, Kapust and Waugh, 1999). Perhaps the presence of the organellar targeting signals leads to these altered folding conformations. In the case of the lupin tRNA nucleotidyltransferase without the mitochondrial/chloroplast targeting signal, perhaps it is the presence of disulfides bonds which leads to the inactive protein. Since this enzyme functions in the cytosol (a reducing environment) perhaps the addition of a small amount of a reducing agent such as dithiothreitol or glutathione to the enzyme assay could have addressed this problem. In this context, it is interesting that the *Arabidopsis* tRNA nucleotidyltransferase lacking its targeting signal and containing only four cysteine residues was active. Similarly, the yeast tRNA nucleotidyltransferase contains only four cysteine residues and has been isolated in our lab in an active form from an *E. coli* expression system (Xunying Shan, personal communication). In spite of the lack of activity from three of the four enzymes, all four of the tRNA nucleotidyltransferase proteins (plus and minus targeting signals) were used for further experiments.

4.1.2 AraGim1p and AraYml079wp

AraGim1p and AraYml079wp were produced as polyhistidine tagged proteins. Using the recombinant protein solubility prediction program described above, both AraGim1p and AraYml079wp are predicted to be about 50% soluble when over-expressed in *E. coli*. Again, this did not agree with what was observed. The AraGim1p was located primarily in the soluble fraction, while the AraYml079wp was localized in the insoluble fraction (Figure 14). Using the Kyte and Doolittle formula (Kyte and Doolittle, 1982) to calculate the average hydrophilicity, it showed that AraGim1p (-0.78) was more hydrophilic than AraYml079wp (-0.16) suggesting that AraGim1p would be about four times more soluble than AraYml079wp. This is in good agreement with what was seen experimentally (Figure 17). Based on the results obtained with all of these proteins, size might be another factor that influences the solubility of the recombinant protein. There is a clear correlation between size and solubility with the smaller proteins, in general, being more soluble than the larger proteins. Clearly, size is not the only criterion though as the two large tRNA nucleotidyltransferases show different levels of solubility.

4.2. Protein-protein interactions

Three methods, cross-linking, far Western blotting and co-elution, were used in this study in an attempt to support the interactions seen in the yeast two-hybrid system. According to the yeast two-hybrid system data, AraGim1p can interact only with the *Arabidopsis* tRNA nucleotidyltransferase containing its mitochondrial/chloroplast targeting signal while AraYml079wp can interact only with the lupin tRNA nucleotidyltransferase containing its mitochondrial/chloroplast targeting signal (Gu, 2000). The results of the interactions shown in this study are summarized in Tables 6 and 7.

4.2.1 *Arabidopsis* tRNA nucleotidyltransferase

Based on the yeast two-hybrid data an interaction between *Arabidopsis* tRNA nucleotidyltransferase containing its mitochondrial/chloroplast targeting signal and AraGim1p was expected. This was supported by the far Western data when AraGim1p was used as the probe protein in the far Western blot (Figure 20, panel C). In addition and unexpectedly, based on the yeast two-hybrid data, a far Western interaction also was seen between the *Arabidopsis* tRNA nucleotidyltransferase containing its mitochondrial/chloroplast targeting signal and AraYml079wp (Figure 20, panel B). In

both of these cases the interaction was specific for tRNA nucleotidyltransferase containing its amino-terminal organellar targeting signal suggesting a role of the targeting signal in this interaction. The unexpected interaction between AraYml079wp and *Arabidopsis* tRNA nucleotidyltransferase (with its targeting signal) could simply be an artifact resulting from too much tRNA nucleotidyltransferase protein on the blot such that this interaction simply represents non-specific association of the two proteins. While the cross-reactivity of AraYml079wp with the molecular weight markers may support this hypothesis, the fact that no interaction with the lupin tRNA nucleotidyltransferase loaded in similar amounts argues against this. Similarly, the specificity of this interaction for tRNA nucleotidyltransferase containing amino-terminal targeting sequences indicates that this is a real interaction. Perhaps, as discussed previously, the targeting signal prevents proper folding of the proteins and either the AraGim1p or AraYml079wp interaction is with any protein that is not folded properly. This idea is not supported for AraGim1p which does not interact with the lupin tRNA nucleotidyltransferase lacking its targeting signal. If one argues that the tRNA nucleotidyltransferases containing their targeting signals also are unfolded in the co-elution experiments then the lack of interaction shown with AraYml079wp (Figure 23) also does not support this hypothesis.

In the far Western assays both AraGim1p and AraYml079wp contained the same His-tags used to purify the proteins. While it seems unlikely that the His-tagged regions of the fusion proteins are responsible for the interaction with the *Arabidopsis* tRNA nucleotidyltransferase (containing its targeting signal), the role of the His-tag in these interactions can not be completely excluded. Perhaps, the targeting signal of *Arabidopsis* tRNA nucleotidyltransferase interacts with the His-tagged portion of both probe proteins. Based on the composition of the His-tag (MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS) and the general character of the targeting signal (basic, hydroxylated and hydrophobic residues) this is probably not the case. The co-elution results using *Arabidopsis* tRNA nucleotidyltransferase do not support an interaction between the His-tagged region of either of these protein and tRNA nucleotidyltransferase.

The *Arabidopsis* tRNA nucleotidyltransferase with or without its mitochondrial/chloroplast targeting signal did not show any interactions in the co-elution experiments (Figures 23 and 24). As discussed above, this may be due to improper folding of the tRNA nucleotidyltransferase. It also is possible that AraGim1p, which based on the two-hybrid and far Western data was expected to interact, was not in its native conformation as it was bound to the column by its His-tag. The attempts at

co-elution also may have been unsuccessful if any real interaction between the proteins involves the amino-terminal end of AraGim1p that may have been hindered by binding to the cobalt column. Similarly, if the His-tagged region of the molecule was involved in an artifactual interaction, this interaction would not be seen in this instance as the His-tagged portion of the protein is bound to the column. In yeast, Gim1p is part of a large complex, GIMC (Geissler *et al.*, 1998). Therefore, other proteins or co-factors may be required for AraGim1p and *Arabidopsis* tRNA nucleotidyltransferase to show an interaction by co-elution. While these co-factors may be present in yeast nucleus to allow a positive two-hybrid interaction, the positive result with the far Western experiment argues against the requirement for a protein complex, or the far Western results represent an artifact.

Glutaraldehyde is a homobifunctional cross-linking reagent which will react with amino groups of proteins. In the cross-linking experiments, when glutaraldehyde was used as the cross-linker, it allowed the tRNA nucleotidyltransferases to cross-link with themselves (Figure 25). *Arabidopsis* tRNA nucleotidyltransferase was present as monomers in the absence of cross-linking reagent (Figure 26). However, in the presence of glutaraldehyde, tRNA nucleotidyltransferase formed a large oligomeric complex (Figure 25). The molecular weight of this higher order form is to be determined.

However, this oligomer of tRNA nucleotidyltransferase confused the results of the cross-linking between tRNA nucleotidyltransferase and AraGim1p or AraYml079wp. Therefore, no firm conclusions can be drawn from the cross-linking experiments using glutaraldehyde.

EDC represents a zero order cross-linker. This reagent induces direct joining of two intrinsic chemical groups of proteins without the introduction of any extrinsic material (Wong, 1991). Since no spacer is introduced, zero-length cross-linking provides a better chance that the linked residues are located in the actual site(s) of protein-protein interaction. EDC is the reagent that condenses the carboxyl groups of glutamic acid or aspartic acid residues on the protein with amino groups to form amide bonds. When EDC and NHS were used as cross-linkers, no self cross-linking was observed (Figure 26). Therefore, tRNA nucleotidyltransferase did not have carboxyl groups and amino groups close enough to allow self cross-linking to occur. No interaction was observed in the case of the *Arabidopsis* tRNA nucleotidyltransferase when EDC and NHS were used as cross-linking agents. This means that no carboxyl group of AraGim1p or AraYml079wp was in close contact with the amino group of *Arabidopsis* tRNA nucleotidyltransferase.

The cross-linking results do not support the observation from the yeast two-hybrid assay. The problem might lie on the nature of the cross-linking techniques. Since the cross-linking techniques detect nearest neighbors, if one cross-linking agent fails to detect the interaction between two proteins, it could mean either that the proteins do not interact or that no functional group is nearby. Since glutaraldehyde as a cross-linker gave no clear interaction, and EDC and NHS contradicted the yeast two-hybrid assay result, more cross-linkers should be used in order to provide a more concrete answer.

Negative results from the co-elution experiments (which seemed to work for AraYml079wp and lupin tRNA nucleotidyltransferase) and from the chemical cross-linking suggest that the interaction between *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p is not biologically relevant. Perhaps any real (biologically relevant) interactions with Gim1p require the presence of a complete GIM complex. This raises the question of why the interaction was observed in the yeast two-hybrid assay. Perhaps Gim1p alone is able to interact with any unfolded or misfolded protein, but can not exert its true role unless associated with the GIM complex. One problem with the yeast two-hybrid assay is that the two proteins have to be directed to the nucleus for the assay to work. There is no evidence that the remaining members of the GIM complex would be localized to the nucleus (Huh *et al.*, 2003). This

proposed non-specific interaction with unfolded proteins also may manifest itself in the results seen with the far Westerns (Figure 20 shown here) and the other interactions studies (Geissler *et al.*, 1998, Uetz *et al.*, 2000, Ito *et al.*, 2001, Gavin *et al.*, 2002, Huang *et al.*, 2002, Tong *et al.*, 2004) which showed multiple interactions with many different types of proteins.

One common characteristic of proteins that give two-hybrid system false positives is that they are intrinsically sticky (Serebriiskii *et al.*, 2000). Those proteins often bind non-specifically to a large number of different proteins or because of nonspecific charge or coiled coil interactions (Serebriiskii *et al.*, 2000). Using the GOR4 secondary structure prediction results (Network Protein Sequence Analysis, http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html), AraGim1p shows 88% alpha helix. This sequence characteristic (extended helical sequences) is thought to promote sticky behavior (Serebriiskii *et al.*, 2000). Previous experiments, (*e.g.*, synthetic lethality, affinity precipitation and two-hybrid system) have shown one hundred sixty-six yeast proteins associated with the yeast Gim1p (Geissler *et al.*, 1998, Uetz *et al.*, 2000, Ito *et al.*, 2001, Gavin *et al.*, 2002, Huang *et al.*, 2002, Tong *et al.*, 2004). Surprisingly, none of these experiments identified yeast tRNA nucleotidyltransferase interacting with Gim1p. This might suggest either that the interaction we observed

between the plant proteins was an artifact of producing these proteins in yeast or that the interaction is specific for the *Arabidopsis* tRNA nucleotidyltransferase and Gim1p homologues. Based on the results presented here, the interaction between *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p shown by yeast two-hybrid is not supported by co-elution and cross-linking and is complicated by far Western data. These results suggest that the two-hybrid interaction between tRNA nucleotidyltransferase and AraGim1p is an artifact, but more experiments may help to define if this reaction is biologically relevant.

4.2.2 Lupin tRNA nucleotidyltransferase

The lack of a yeast two-hybrid interaction between lupin tRNA nucleotidyltransferase (containing its mitochondrial/chloroplast targeting signal) and AraGim1p was supported when it was used as the probe protein in the far Western blot (Figure 20, panel C) and by the co-elution experiment (Figure 22). Moreover, neither AraGim1p nor AraYml079wp interacted with lupin tRNA nucleotidyltransferase (with or without its targeting signal) when AraGim1p or AraYml079wp was used as a probe in far Western analysis (Figure 20, panels B and C). This is unexpected as AraYml079wp

was shown to interact with lupin tRNA nucleotidyltransferase (containing its targeting signal). Interestingly though, when lupin tRNA nucleotidyltransferase containing its targeting signal is used as a probe in a far Western blot it does show an interaction with AraYml079wp (Figure 19) as expected. It is unclear why a far Western blot interaction would be seen when lupin tRNA nucleotidyltransferase was used as a probe to AraYml079wp on the membrane while no signal was seen when the reciprocal experiment was performed. Perhaps lupin tRNA nucleotidyltransferase is denatured when immobilized on the filter but folds correctly in solution and the interaction with AraYml079wp can only happen when the lupin tRNA nucleotidyltransferase is folded correctly.

The co-elution data support an interaction between lupin tRNA nucleotidyltransferase (with its organellar targeting signal) and AraYml079wp but not with AraGim1p (Figure 21 and 22). Furthermore, no interaction was observed with either protein when lupin tRNA nucleotidyltransferase lacks its organellar targeting signal (Figure 21 and 22). These results are in good agreement with the two-hybrid data and suggest a role for the N-terminal targeting signal of lupin tRNA nucleotidyltransferase in this interaction. Moreover, these results support the far Western data when lupin tRNA nucleotidyltransferase is used as a probe (Figure 19).

As with the *Arabidopsis* tRNA nucleotidyltransferase, the lupin tRNA nucleotidyltransferase was present as monomers in the absence of cross-linking reagent (Figure 26). However, in the presence of glutaraldehyde, lupin tRNA nucleotidyltransferase formed a large oligomeric complex (Figure 25). Therefore, no firm conclusions can be drawn from the cross-linking experiments using glutaraldehyde. Again like the *Arabidopsis* tRNA nucleotidyltransferase experiments, when EDC and NHS were used as cross-linkers, no self cross-linking of lupin tRNA nucleotidyltransferase was observed (Figure 26). These experiments did show that the lupin tRNA nucleotidyltransferase (with or without its mitochondrial/chloroplast targeting signal) could interact with both AraGim1p and AraYml079wp when lupin tRNA nucleotidyltransferase was added first to the reaction. This means that a carboxyl group of the lupin tRNA nucleotidyltransferase was in close contact with an amino group of both AraYml079wp. A high molecular weight species was formed when cross-linking reagent was added (Figure 26). Therefore, in this case, the targeting signal was not important for this interaction to occur. The ratio of lupin tRNA nucleotidyltransferase to AraYml079wp required to form the complex and the exact molecular weight of the oligomer needs to be determined.

In contrast to Gim1p, which seems to interact with very many proteins in yeast, Yml079wp was shown to interact only with the *ATP14* gene product (Ito *et al.*, 2001). The *ATP14* gene product also contains a mitochondrial targeting signal (Arselin *et al.*, 1996). These findings taken together with the results obtained here suggest that the interaction between lupin tRNA nucleotidyltransferase and AraYm079wp might be specific and biologically relevant. More research will be required to determine what role AraYml079wp plays in lupin in the synthesis, folding, targeting, transport or function of tRNA nucleotidyltransferase.

4.3. Characterization of the yeast *YML079w* deletion

AraYml079wp was a novel protein identified from the yeast two-hybrid assay. It was the only protein that interacted with the lupin tRNA nucleotidyltransferase containing its organellar targeting signal. The predicted function of AraYml079wp is currently unknown even though the gene is conserved in many organisms. While homologues are evident in many prokaryotic genomes (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi BLASTP 2.2.7 [Jan-05-2004], RID: 1073535068-21463-93299433326.BLASTQ4), only a few eukaryotes (*Arabidopsis*, *Oryza sativa, Saccharomyces cerevisiae, Aspergillus nidulans* FGSC A4, *Branchiostoma*

belcheri tsingtaunese, *Neurospora crassa*) have easily identifiable homologues. The yeast Yml079wp has about 26% sequence identify to this novel *Arabidopsis* gene product (Figure 9). Since the *YML079w* deletion strain is viable this indicates that this gene is not essential in yeast (Giaever *et al.*, 2002). In fact, this protein has no known function in yeast. Using a green fluorescent protein fusion protein, Yml079w was found localized to the nucleus and cytoplasm (Huh *et al.*, 2003). Molecular interaction data from an organism-wide screen of *Saccharomyces cerevisiae* suggest that the *YML079w* gene product only interacts with the yeast *ATP14* gene product (one of the subunits of the mitochondrial ATP synthase) (Ito *et al.*, 2001).

To understand more about the function of this gene, a number of simple experiments were carried out. For example, since Yml079wp interacts with the *ATP14* gene product and tRNA nucleotidyltransferase (two proteins that contain mitochondrial targeting signals) the ability of the deletion strain to grow on glycerol (a non-fermentable carbon source) was tested. Deletion of the *YML079w* gene does not affect the ability of yeast to grow on glycerol (Figure 27) indicating that the *YML079w* deletion strain is still capable of mitochondrial respiration and that the *YML079w* gene product is not required to target the *ATP14* gene product or tRNA nucleotidyltransferase to the mitochondrion.

It was equally easy to determine if the *YML079w* gene product was necessary for growth at high or low temperatures. Without this gene, there was no temperature-sensitivity or cold-sensitivity in the yeast cell (Figure 27). Temperature-sensitive mutants often result from missense mutations in genes coding for structural proteins and sometimes are located in genes coding for tRNA species (Pringle, 1975). Cold-sensitive mutants are frequently defective in genes whose protein products must have allosteric functions or participate in assembly of complex structures (Pringle, 1975). For example, deletion of the yeast *GIM1* gene, a part of a major complex involved in tubulin formation, results in a cold-sensitive phenotype (Geissler *et al.*, 1998). Since the *YML079w* gene deletion did not result in the production of a temperature or cold-sensitive mutant, this suggests that the *YML079w* gene is not involved in these types of functions.

While replica plating results indicated that the *YML079w* deletion strain can grow on rich media, this does not address directly any effect the deletion may have on growth rate or on the ability to leave lag phase. To test if the deletion has an effect on the exponential grow phase, growth curves comparing the growth rates of yeast with or without *YML079w* were generated. According to the growth curves obtained (Figure 28), there was no effect observed on doubling times. The effect of the *YML079w*

deletion on long term viability also was tested. Again, without this gene, cells remain viable on both fermentable and non-fermentable carbon sources to the same extent as the wild-type control (Figure 29 and 30).

From the microarray data (Gasch *et al.*, 2000), when 0.32 M hydrogen peroxide was added to yeast cell cultures, the expression of the *YML079w* gene increased one-fold after 50 minutes. Therefore, to further examine the role that the *YML079w* gene played in the response to hydrogen peroxide, different concentrations of hydrogen peroxide were added to cells with or without the *YML079w* gene. There is no difference between the deletion strain and the isogenic control strain after treatment with hydrogen peroxide (up until 1mM). The cells can still grow well on glucose or glycerol medium (Figures 31 and 32). Therefore, at low concentrations of hydrogen peroxide, the respiration of the yeast cell is not effected. However, above 2 mM hydrogen peroxide most of the wild-type and *YML079w* mutant cells were dead. Therefore, the *YML079w* gene did not protect the cells from oxidative stress.

Cells without the *CCP1* gene were found to be more susceptible to hydrogen peroxide-induced stress during their exponential and stationary phases of growth (Shephard, 1999). Therefore, this strain was used as a positive control in this experiment. As expected, after treatment with hydrogen peroxide, the *CCP1* deletion

strain had an IRC about 20% lower than the wild type although the *YML079w* deletion strains showed no significant difference from wild type (Figure 32).

From the results obtained, the *YML079w* gene deletion did not affect cell doubling time, long term growth rate, respiratory competence, hydrogen peroxide stress sensitivity, or the ability of the cells to grow on fermentable or non-fermentable carbon sources, or at high or low temperatures. Therefore, the role of the yeast *YML079w* gene and of its *Arabidopsis* homologue are still undetermined.

Conclusions and future work

In this study, three different *in vitro* methods were used to try to reproduce, *in vitro* two interactions identified *in vivo* by the yeast two-hybrid system. The *in vitro* data (co-elution, far Western and cross-linking) seemed to support fairly well the interaction seen between the lupin tRNA nucleotidyltransferase and AraYml079wp suggesting that this interaction may have biological significance. In contrast, the interaction between the *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p was not supported by the co-elution and cross-linking experiments. Moreover, in the far Western experiment AraGim1p showed unexpected interactions suggesting that this protein is subject to non-specific interactions with various proteins. Taken together these results suggest that the biological relevance of the yeast two-hybrid interaction seen between *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p might be questionable.

Clearly, more needs to be done to confirm whether the interaction between the *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p is biologically relevant or an artifact. For example, it would be interesting to see if yeast Gim1p interacts with *Arabidopsis* tRNA nucleotidyltransferase in the yeast two-hybrid system. To prove more conclusively that the AraYml079wp interaction with lupin tRNA nucleotidyltransferase is biologically relevant more experiments could be performed.

For example, colocalization, immunoprecipitation or cell fractionation could be used to show that these proteins are found together *in vivo*.

Many proteins could be involved in tRNA nucleotidyltransferase production, maturation and localization; however, previous studies in yeast identified only enolase (Gavin *et al.*, 2002) and a single-stranded DNA endonuclease (Uetz *et al.*, 2000) as interacting with this protein. Other methods such as synthetic lethality could be used to find more proteins interacting with tRNA nucleotidyltransferase in plants.

Since AraYml079wp is novel protein, its function is still unknown. A number of experiments were performed to find a function for Yml079wp, but the lack of this gene product showed no detectable phenotype under any of the conditions tested. Therefore, the function of the Yml079wp still needs to be determined.

Perhaps the *YML079w* gene product exerts its effects in combination with other gene products, for example, *yap1*. According to the microarray data, the expression of *YML079w* increased 2 fold when *yap1* was over-expressed (Gasch *et al.*, 2000). In a *yap1* deletion strain, the expression of *YML079w* also increased two-fold after adding 0.3 mM hydrogen peroxide for 20 minutes. Therefore, a double *YML079wp/YAP1* deletion might be of use to study the function of Yml079wp. Synthetic lethality or using AraYml079wp itself in a yeast two-hybrid assay might also be good tools to identify if

other proteins interact with Yml079wp. Sometimes, deletion of a single gene has no obvious phenotype, but its over-expression confers lethality (Liu *et al.*, 1992). Therefore, it might be interesting to see if this is the case of the *YML079w* gene.

In conclusion, *in vitro* results using co-elution and far Western blotting supported the *in vivo* results for an interaction between lupin tRNA nucleotidyltransferase and AraYml079wp. However, the interaction between *Arabidopsis* tRNA nucleotidyltransferase and AraGim1p does not appear biologically relevant and may represent an artifact. In addition, a number of experiments were performed using a yeast *YML079w* deletion strain to try to elucidate a function for Yml079wp. However, no detectable phenotype was found. Therefore, the role of the yeast *YML079w* gene and of its *Arabidopsis* homologue are still undetermined.

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