Characterization of new putative amino acid transporters of the ATF1 superfamily in *Arabidopsis thaliana*

Dissertation

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Contents

AAP	Amino acid Permease
ABC transporter	ATP Binding Cassette transporter
ATF superfamily	Amino acid Transporter superfamily
BLAST	Basic Local Alignment Search Tool
CAT	Cationic Amino acid Transporter
CSM	Complete Supplement Mixture
Cv	Cultivar
DOB	Drop Out Base
DON	6-diazo-5-oxo-norleucine
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
GABA	Gamma Amino Butyric Acid
GFP	Green Fluorescent Protein
LB	Luria Broth
NCBI	National Center for Biotechnology Information
PABA	Para Amino Benzoic Acid
YNB	Yeast Nitrogen Base
YPD	Yeast extract Peptone Dextrose medium
SC	Synthetic Complete complements or medium
SD	Synthetic Defined medium
ssDNA	single strand Deoxyribonucleic acid
Tris	Tri(hydroxymethyl)aminomethane
v/v	volume for volume
w/v	weight for volume
X-Gluc	5-bromo-4-chloro-3-indolyl-α-galactopyranoside
YNB	Yeast Nitrogen Base

Summary

The uptake of nitrogen and its translocation in the plant involve the transport of organic nitrogen in the form of amino acids. During the last years, the focus of the studies on amino acid transport in plants moved from biochemical to molecular characterization of the carrier proteins required for this process. Not all the predicted transport fluxes through the membranes of plant cells have already been assigned to a particular carrier protein, although many carrier proteins were functionally characterized. Moreover, many other proteins, whose existence was predicted by genome sequencing and large scale EST projects, were suggested to function as amino acid transporters on the base of sequence similarity and predicted structural features. However, many of such putative transport proteins have not yet been functionally characterized.

In this study a group of related *Arabidopsis* genes encoding putative amino acid transporters have been analyzed in detail. The *in silico* analysis grouped these genes to the ATF1 superfamily, suggesting a function as amino acid transporters. These proteins have been called AT, for <u>A</u>mino acid <u>T</u>ransporters.

Attempts to study these proteins by heterologous expression in *Saccharomyces cerevisiae* and complementation of yeast mutants defective in amino acid uptake, did not allow their functional characterization.

The AT proteins are closely related to the yeast AVTs, that have been described as vacuolar transporters of amino acids. Thus, a hypothesis was raised that the AT proteins act as intracellular transporters of amino acids, located on the tonoplast. A yeast mutant strain, G119, was constructed to study vacuolar asparagine transporters which requires further optimization.

The subcellular localization of AT1 and AT12 was determined by fusions with GFP and expression in *Arabidopsis* protoplasts. In these assays, both AT1 and AT12 were localized in endomembranes. Therefore, an alternative hypothesis on the function of the AT proteins was postulated: they may act as vesicular transporters of amino acids, involved in export processes from the cell, in a similar way to the mechanism suggested for the phylogenetically related RnVGAT, exporter in rat synapsis.

To provide evidence for the possible physiological processes in which these proteins are involved, the activity of the promoter of AT4, AT5, AT9 and AT12 was analysed by promoter-GUS fusions. AT4 was expressed in the hypocotyl and in the petiole of young leaves and might play a role during the rapid cell elongation found in these organs. AT5 was

not expressed under standard growth conditions. AT9 and AT12 were expressed in cotyledons and young leaves, suggesting a role in the supply of nitrogen to sink organs in young seedlings. They were also expressed in the pollen, therefore these proteins might have a role in the gametogenesis. AT9 was in addition also expressed in roots of developing seedlings and in the lateral roots of adult plants. A role of AT9 in the uptake of amino acids from the soil can therefore be postulated.

The analysis of T-DNA insertion lines has revealed that the knock-out mutants in the AT7 gene are sterile. This might suggest an essential role of AT7 in the gametogenesis.

Zusammenfassung

Die Aufnahme und Verteilung von Stickstoff in Pflanzen beinhaltet den Transport von organischem Stickstoff in Form von Aminosäuren. In den letzten Jahren hat sich der Schwerpunkt der Forschung im Bereich des Aminosäuretransports von biochemischen Studien in Richtung der molekularen Charakterisierung an diesem Prozess beteiligter Transportproteine verschoben. Noch wurden nicht alle vorhergesagten Transportströme durch die Zellmembranen von Pflanzen einem bestimmten Protein zugeordnet. Viele Transportproteine wurden funktionell charakterisiert. Vielen anderen Proteinen, deren Existenz durch genomisches Sequenzieren und EST Projekten vorhergesagt wurde, kann aufgrund von Sequenzenähnlichkeiten und strukturellen Merkmalen eine Funktion als Aminosäuretransporter zugeordnet werden. Viele dieser Proteine sind zurzeit noch nicht charakterisiert.

In dieser Arbeit wurden einige Gene aus *Arabidopsis* detailliert analysiert, die vermeintlich Aminosäuretransporter kodieren. Die *in silico* Analyse ermöglichte, diese Gene der ATF1-Überfamilie zuzuordnen und eine Funktion als Aminosäuretransporter vorherzusagen. Diese Proteine wurden AT genannt, für <u>A</u>minosäure<u>t</u>ransporter.

Versuche, die Funktion dieser Proteine durch heterologe Expression in Saccharomyces cerevisiae und Komplementation von Hefemutanten, deren Aminosäureaufnahme gestört war, zu bestimmen, haben keine funktionelle Charakterisierung ermöglicht.

Die AT Proteine sind eng verwandt mit den AVTs in Hefe, die als vakuoläre Aminosäuretransporter beschrieben wurden. Eine Hypothese zur Funktion der Gene ist also, dass die AT-Proteine als intrazelluläre Aminosäuretransporter agieren und im Tonoplasten lokalisiert sind. Eine Hefemutante, G119, wurde hergestellt, um vakuolären Asparagintransportern zu untersuchen was weiterer Optimierung bedarf.

Die subzelluläre Lokalisierung von AT1 und AT2 wurde durch Fusionen mit GFP und Expression in *Arabidopsis* Protoplasten bestimmt. Diese Untersuchungen zeigten, dass AT1 und AT12 in Endomembranen lokalisiert sind. Daher wurde eine alternative Hypothese der Funktion dieser Proteine aufgestellt: Sie könnten als vesikuläre Aminosäuretransporter am Exportsystem der Zelle beteiligt sein, ähnlich dem Mechanismus, der für die phylogenetisch verwandten RnVGAT, Exporter in Rattensynapsen, vorgeschlagen wurde.

Um Beweise für die möglichen physiologischen Prozesse zu finden, an denen diese Proteine beteiligt sind, wurde die Promotoraktivität von AT4, AT5, AT9 and AT12 durch Promotor-GUS-Fusionen untersucht. AT4 wurde im Hypokotyl und in den Petiolen junger Blätter

exprimiert und könnte während der schnellen Zellstreckung dieser Organe eine Rolle spielen. AT5 wurde unter Standardbedingungen nicht exprimiert. AT9 und AT12 werden in Keimblättern und jungen Blättern exprimiert, was für eine Rolle bei der Stickstoffversorgung von Sinkorganen junger Keimlinge spricht. Sie werden außerdem im Pollen exprimiert und könnten eine Rolle bei der Gametogenese spielen. AT9 wird zusätzlich in Wurzeln sich entwickelnder Keimlinge und in den Lateralwurzeln adulter Pflanzen exprimiert. Daher kann eine Rolle bei der Aufnahme von Aminosäuren aus dem Boden postuliert werden.

Die Analyse von T-DNA-Insertionslinien zeigt, dass Linien, die eine Knock-Out-Mutation im AT7-Gen tragen, steril sind. Das spricht für eine essentielle Rolle dieses Gens während der Gametogenese.

1   Introduction

1.1. Nitrogen acquisition by the plant

Nitrogen is an essential element in plant nutrition, as it is part of fundamental molecules such as nucleic acids and proteins, as well as many other compounds. Nitrogen is often the limiting factor of plant growth. Nitrogen is acquired by the plant through compounds taken up by the roots, with the only exception of carnivorous plants that supply their nitrogen reserve by digestion of insects (Albert *et al.*, 1992).

A variety of compounds serve as nitrogen sources in soil. The major forms in which it is acquired are the inorganic compounds ammonium and nitrate. However, some soils contain amino acids as well as small peptides at high concentrations and might serve as an important source of nitrogen (Chapin *et al.*, 1993, Näsholm *et al.*, 1998, Persson & Näsholm, 2001).

The acquisition of nitrogen by the plant follows different paths. Many plants use symbiosis with bacteria or fungi. In the case of bacterial symbiosis (Udvardi & Day, 1997), ammonia is released in infected cells of the plant, then assimilated and translocated to other plant organs; the association of plant roots and fungi, that facilitate the acquisition of nitrogen from the soil, is called ectomychorriza (Chalot & Brun, 1998).

1.2. Nitrogen translocation in the plant body

Following the carrier-mediated uptake of nitrate or ammonia from soil, inorganic nitrogen is assimilated, i.e. used to synthesize organic compounds, usually amino acids in an energy-requiring process (Von Wiren *et al.*, 1998). This assimilation process can occur differently depending on the form of inorganic nitrogen that has been acquired and plant species. The assimilation of ammonia usually takes place in the roots, while nitrate can be assimilated by the photosynthetic active leaf cells after translocation via the xylem or in other cases by the roots, depending on the environmental conditions and the plant species (Andrews, 1986).

The main transport form of organic nitrogen in most plant species are amino acids. After the assimilation of nitrogen in organic compounds in the root, amino acids are translocated via the xylem. When nitrogen is assimilated in the leaves, excess amino acids are translocated via the phloem and can be exported to the roots (Marschner *et al.*, 1997). Nitrogen is also exchanged between xylem and phloem in the root under conditions of abundant nitrogen supply (Cooper

& Clarkson, 1989). Therefore, amino acids can be found both in the xylem and in the phloem and can be withdrawn from the vascular system by cells dependent on an external supply of nitrogen, such as the apical meristems, newly developing tissues and the reproductive organs. Seeds are loaded with nitrogen in the form of amino acids through the phloem, demonstrating that there must be transport from the xylem (Pate *et al.*, 1977).

The spectrum of amino acids found in the xylem is similar in most plant species to the one found in the phloem, as well as to the amino acid composition of mesophyll cells (Lohaus *et al.*, 1995, Riens *et al.*, 1991). The major form present in phloem and xylem are amides, such as glutamine and asparagine. The relative concentration of amino acids differs very much: the phloem contains high concentrations, e.g. 60-140 mM in sugar beet (Lohaus *et al.*, 1994), while the xylem contains lower concentrations, e.g. 3-20 mM in *Urtica dioica* (Rosnick-Shimmel, 1985). Because of the loading of amino acids from the xylem to the phloem, the composition of the phloem sap can differ along its transport path (Pate, 1980).

1.3. Transport of amino acids in the plant

Amino acids are produced and transported following the assimilation of inorganic nitrogen, but they also derive from the breakdown of storage proteins in vegetative and reproductive storage organs, supplying the plant during its development with nitrogenous compounds.

The long distance amino acid transport requires carrier proteins. The transport of amino acids in the plant and the processes in which an amino acid carrier is required are schematized in Figure 1. There are physiological and genetic evidences for the activity of multiple carrier-mediated transport systems (Bush, 1993). When the amino acids are loaded in the phloem, a carrier is required in the case of apoplastic phloem loading system. In the case of translocation into the seeds, a carrier is also required: import into the embryo requires two transport steps, one for release from the seed coat and one for uptake into the endosperm and embryo (Pate *et al.*, 1977). When amino acids are loaded into the xylem, or unloaded from it they must cross the plasma membrane of the source cells or of the xylem parenchyma, as the tracheis elements are dead cells and symplastic transfer is not possible. Exchanges between xylem and phloem, demonstrated by the fact that amino acids are found unchanged in the phloem once loaded in the xylem (Atkins, 2000, Pate & Sharkey, 1975), also require the presence of carrier proteins. Plants also possess the ability to take up amino acids from the soil through the root, which

requires an active transport through carrier proteins present on the root surface (Persson & Näsholm, 2001).
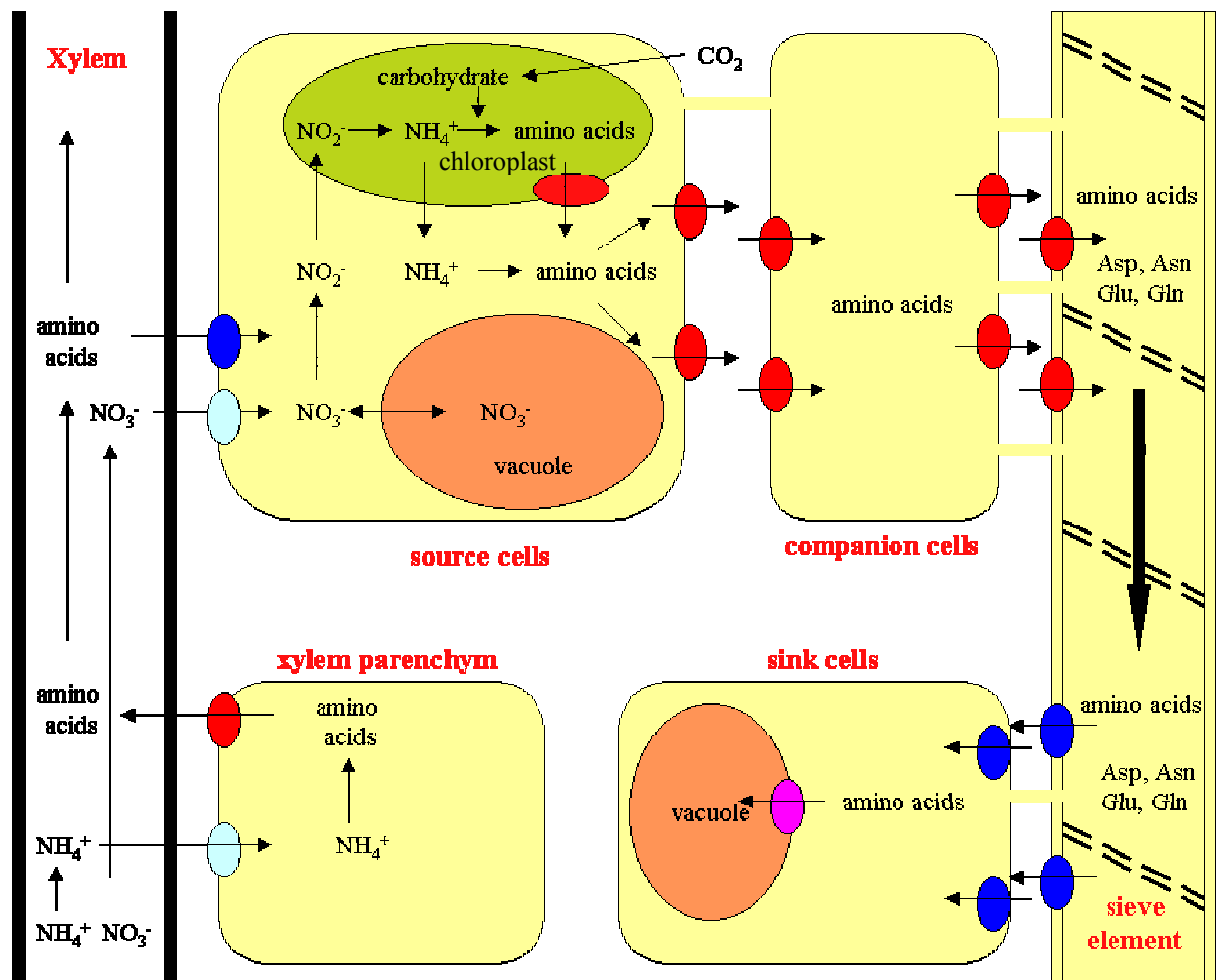
Figure 1 – Transport of nitrogenous compounds in the plant.

1.4. Intracellular transport of amino acids

Carriers are also required to mediate intracellular amino acid transport. Chloroplasts are the primary compartment of amino acid biosynthesis (Lam *et al.*, 1996), but the biochemical pathways for synthesis and degradation of amino acids also involve other organelles like mitochondria and peroxysomes (Miflin & Lea, 1982). Therefore, several membranes must be crossed by carrier-mediated transport.

The vacuole contains amino acids and functions as a storage compartment, as well as a lytic environment in which amino acids are released from the breakdown of storage proteins. The

composition of amino acids in the vacuole is different from the cytoplasm. In the vacuole of mesophyll cells of barley primary leaves a prevalence of alanine, glutamine and leucine has been measured (Dietz *et al.*, 1989). The quantity of amino acids present in the vacuole is variable and depends on the environmental conditions, but it represents in any case an important percentage of the total free amino acids present in the cell (Martinoia & Ratajczak, 1997). Three different carrier-mediated transporter systems have been predicted on the tonoplast for the import of amino acids: a proton-motive force dependent aromatic amino acids transport system, a positive-charged amino acids transport system and a broad specificity ATP-regulated, not energy-requiring transport system. The existence of one or more energy requiring mechanisms for the export of amino acids from the vacuole in the cytoplasm has been also postulated (Martinoia *et al.*, 1991).

The existence of mitochondrial carriers for amino acids has been predicted, for example in the case of the proline / glycine carrier (Laloi, 1999). Two related mitochodrial carrier proteins, AtmBAC1 and AtmBAC2, both arginine-ornithine translocators, have been also isolated and characterized at a molecular level (Catoni *et al.*, 2003, Hoyos *et al.*, 2003).

1.5. Molecular characterization of amino acid transporters

The first characterized plant amino acid carrier is a broad specificity amino acid permease AtAAP1, isolated by complementation of the proline uptake deficient yeast strain 22457d with a cDNA library of *Arabidopsis thaliana* (Frommer *et al.*, 1993). This opened the way for the characterization of many other amino acid transporters, isolated by similar methods (Fischer *et al.*, 1998). Several other amino acid transporters have been discovered by heterologous screening of cDNA libraries in yeast, in Arabidopsis and in other organisms: AtAAP2 (Kwart *et al.*, 1993), AtAAP3, 4 and 5 (Fischer *et al.*, 1995), AtAAP6 (Fischer *et al.*, 2002), AtAAP8 (Okumoto *et al.*, 2002), AtProT1 and AtProT2 (Rentsch *et al.*, 1996), AtCAT1 (Frommer *et al.*, 1995), AtLHT1 (Chen & Bush, 1997), AtANT1 (Chen *et al.*, 2001), AtmBAC1 and AtmBAC2 (Hoyos *et al.*, 2003) in *Arabidopsis thaliana*, RcAAP1 and RcAAP2 (Bick *et al.*, 1998, Marvier *et al.*, 1998) and RcAAP3 (Neelam *et al.*, 1999) in *Ricinus communis*, NaAAP1 (Schulze *et al.*, 1999) in Nephentes, PsAAP1 and PsAAP2 (Tegeder *et al.*, 2000) in *Pisum sativum*, VfAAP1 and VfAAP2 (Montamat *et al.*, 1999) in *Vicia faba*, StAAP1 (Koch *et al.*, 2003) in *Solanum tuberosum*.

1.6. Amino acid transporters in *Arabidopsis thaliana*

The AAP family of amino acid transporters contains eight members (Fischer *et al.*, 1995, Okumoto *et al.*, 2002). ProT1 and ProT2 (Rentsch *et al.*, 1996) have been isolated using the PLAS23-4B strain, defective in the targeting of the endogenous amino acid permeases. AAT1/CAT1, was discovered by complementation of the JT16 strain, defective in histidine uptake (Frommer *et al.*, 1995). LHT1, lysine and histidine carrier (Chen & Bush, 1997) and ANT1, for aromatic and neutral amino acids (Chen *et al.*, 2001) have been discovered by *in silico* screening of the EST collections and functionally complement the JT16 strain.

The AAP family comprises of both low and high affinity systems and can be subdivided into two subfamilies, according to their substrate specificity, into general amino acid transporters and systems that recognize preferentially neutral and acidic amino acids (Fischer *et al.*, 1995).

AAP1 is able to complement both the JT16 and the 22574d yeast strains. It is a carrier with broad specificity, that has a Km for proline in the order of 60 μM (Frommer *et al.*, 1993) and for alanine of 242 μM (Hsu *et al.*, 1993). Northern blot analysis has shown a high level of expression in developing pods and lower expression in flowers, developing leaves and cauline leaves. The *in situ* hybridization showed expression in the vascular system of the cotyledons and the elongation zone adjacent to the root meristem (Kwart *et al.*, 1993).

AAP2 is able to complement the inability of the 22547d yeast strain to grow on citrulline or proline as the sole nitrogen source and the inability of the JT16 yeast strain to grow on low levels of histidine. It is a broad specificity carrier. Expression of the gene has been detected in developing pods and stems and to a lower extent in flowers, roots and developing leaves (Kwart *et al.*, 1993).

AAP3, 4 and 5 complement both the JT16 and the 22574d yeast strains. The Km for proline of these transporters is in the range 100-250 μM. AAP3 is selectively expressed in roots, whereas AAP4 is expressed in leaves, stems and flowers. AAP5 is expressed in all organs with high expression levels in leaves, stems and flowers (Fischer *et al.*, 1995).

AAP6 is an high affinity amino acid transporter. It has been isolated using the yeast strain PLAS23-4B, which carries a mutation in the gene *shr3*, that encodes a protein required for

correct targeting of yeast endogenous amino acid transporters to the plasma membrane; this mutant strain has a reduced amino acid uptake capacity. AAP6 is also able to complement the 22547d strain for its deficiency to grow on proline as sole nitrogen source (Rentsch *et al.*, 1996). It has been localized in the xylem parenchyma, probably acting as an uptake system for amino acids from the xylem, where a high affinity transporter is required, due to the low amino acid concentrations in the xylem sap (Okumoto *et al.*, 2002).

AAP7 is the only member of the AAP family for which functional complementation in yeast has not been achieved.

The AAP8 gene is expressed in siliques and developing seeds, being probably responsible for the loading of organic nitrogen into the seeds. AAP6 and AAP8 are the only members of the AAP family that transport aspartate with an high affinity (Okumoto *et al.*, 2002).

CAT1 is able to complement both the JT16 and the 22574d yeast strains. The preferential substrate is proline, followed by glutamic acid, valine, glutamine, histidine, arginine and lysine, with a Km for histidine of 35 μM. Gene expression has been detected by northern blotting in leaves, roots, flowers and developing siliques. Promoter analysis has shown GUS activity in various floral tissues and in the vascular system of leaves and roots (Frommer *et al.*, 1995). CAT1 shares significant structural and sequence homologies with the mammalian CAT family of amino acid transporters (Kim *et al.*, 1991). It is the only functionally characterized plant transporter that does not belong to the ATF1 superfamily but to the APC superfamily.

LHT1 is an amino acid carrier that complements the yeast strain JT16. It is a high affinity transporter for lysine and histidine, with a Km for lysine of 175 μM and for histidine of 400 μM. It is able to transport a broader range of amino acids with a lower affinity, but under the physiological range, with a Km for leucine of 11 mM. Analysis by northern blotting and *in situ* hybridization revealed a preferential expression in flowers, young leaves, and siliques, but expression is also present in older leaves, stems and roots (Chen & Bush, 1997).

ANT1 is an amino acid transporter, able to complement the JT16 strain. It has been identified by analysis of cDNA clones in EST (Expressed Sequence Tags) libraries that shared a low level sequence similarity with AtAAP1. It is a carrier for aromatic and neutral amino acids,

with a Km for leucine of 163 μM and for tyrosine of 240 μM. It is expressed in all tissues with maximum abundance in flowers and cauline leaves. (Chen *et al.*, 2001).

ProT1 and ProT2 are proline carriers. They were isolated using the PLAS23-4B yeast strain. They also complement the 22547d yeast strain for its inability to grow on proline as sole nitrogen source. The transport activity of proline showed a Michaelis-Menten constant of 360 μM. They are specific carriers for proline, unable to transport any other amino acid efficiently. Northern blot analysis showed that ProT1 and ProT2 are expressed at low level in all organs, with elevated levels in roots, stems and flowers. As proline is an important compound for tolerance towards osmotic stress in plants, the expression of the ProTs was investigated under high-salt stress. It was demonstrated that under these conditions ProT2 expression is increased (Rentsch *et al.*, 1996).

In conclusion, in *Arabidopsis* multiple sets of amino acid transport carriers exist. This is not surprising given the high number of different organs and cell types. The development of such a variety of different transport systems can be explained by considering the differential expression patterns. There is a large number of general amino acid permeases which are required under normal environmental conditions for several processes. These include loading the excess of amino acids into the vascular system, import and export from the seeds, that require a rapid translocation of transiently stored amino acids and the mobilization of amino acids stored in the form of proteins, especially in vegetative organs, during leaf senescence and during germination from cotyledons and endosperm. In contrast, more specific amino acid transporters, such as the ProTs, might be required under defined conditions, like drought or salt stress, where specific amino acids have to be mobilized preferentially (Fischer *et al.*, 1998).

1.7. Yeast as a model organism for the study of amino acid transport

The yeast *Saccharomyces cerevisiae* has been very useful for cloning and expressing plant genes and investigating the substrate specificity of transporters. Several endogenous permeases with different substrate specificities, affinities and regulation exist in this organism. Since the genomic sequencing of *Saccharomyces cerevisiae* has been completed, a nearly complete picture of the amino acid transport systems of this organism is now available. Uptake across the plasma membrane is mediated by 24 different amino acid permeases that

belong to the APC (amino acid-polyamine choline facilitator) superfamily (Wipf *et al.*, 2002). All the yeast members of this family contain 12 putative transmembrane domains and have been functionally characterized. These transporters can be grouped into two subfamilies: the largest group includes permeases that are related to GAP1 (general amino acid permease), like CAN1, an H^+-arginine symporter, HIP1 (histidine permease), PUT4 (proline permease), LYP1 (lysine permease), BAP2 (transporter for aliphatic amino acids), TAT1 and TAT2 (transporters for aromatic amino acids), GNP1 (glutamine permease), DIP5 (transporter for acid amino acids), PAP1 (low affinity permease for branched amino acids) and seven other members. The next group includes proteins which are more related to UGA4, a GABA permease and include MUP1 and MUP3 (methionine permeases), HNM1 (choline permease) and BIO5 (permease of keto-aminopelargonic acid and diaminopelargonic acid) (Fischer *et al.*, 1998).

Five *Saccharomyces cerevisiae* strains deficient in amino acid uptake have been used to identify and characterize plant amino acid transporter genes. Strain 22574d carries mutations in GAP1, PUT4 and UGA4 and is unable to grow on media containing proline, citrulline or GABA as the sole nitrogen source (Jauniaux & Grenson, 1990). Strain 22Δ6AAL was derived from 22574d with a further disruption of CAN1, LYP1, APL1 and LYS2 and depends on either high concentration of lysine or dipeptides containing lysine for its growth (Fischer *et al.*, 2002). Strain 22Δ8AA was derived from 22574d with a further disruption of CAN1, LYP1, APL1, HIP1 and DIP5 and is unable to use also aspartic acid, glutamic acid and arginine as the sole nitrogen source, in addition to the same amino acids as 22547d (Fischer *et al.*, 2002). Strain JT16 carries mutations in HIP1 and CAN1, permeases that transport histidine and HIS4, histidinol dehydrogenase, in the biosynthetic pathway of histidine, and lacks functional histidine uptake and biosynthesis, requiring high concentration of histidine for its growth (Tanaka & Fink, 1985). Strain PLAS23-4B carries a mutation in the SHR3 gene: this gene codes for an integral membrane protein component of the endoplasmic reticulum which is required for the correct targeting in the plasma membrane of all the amino acid permeases; mutations in this gene reduce the rate of amino acid uptake into yeast, as only a reduced amount of amino acid permeases reach the plasma membrane, while the majority accumulate in the ER (Kuehn *et al.*, 1996, Ljungdahl *et al.*, 1992).

1.8. Purpose of this study

The aim of the research project presented in this thesis is to characterize new putative amino acid transporters in order to further understand the complicated overall picture of the amino acid transport in *Arabidopsis thaliana*, in which several proteins have already been identified and functionally characterized as amino acid transporters.

Since the Arabidopsis genome was completely sequenced (Arabidopsis Genome Initiative, 2000), new possibilities were opened for the identification of new amino acid transporters. The yeast proteins ScAVTs have been described as amino acid transporters, located in the tonoplast (Russnak *et al.*, 2001). The existance of several amino acid transport system in the tonoplast of plants have been postulated (Martinoia *et al.*, 1991). However, no plant vacuolar amino acid transporters have been yet identified.

The closest homologs of the ScAVTs in Arabidopsis shall be identified with the use of sequence alignment tools. Predicted gene products shall be assigned to putative functions, on the basis of sequence similarity and other predicted properties, like the presence of putative transmembrane domains.

The new putative amino acid transporters shall be cloned in yeast expression vectors. Yeast mutants deficient in amino acid transport, JT16 (Tanaka & Fink, 1985) and 22Δ8AA (Fischer *et al.*, 2002) shall be used for functional characterization. A new strain shall be developed to identify asparagine transporters localized on the tonoplast. In addition the use of toxic analogs of amino acids shall be used as a tool to identify cell exporters or vacuolar importers of amino acids.

To understand in which physiological processes the new putative amino acid transporters are involved, the activity of the promoters of the these transporters shall be analyzed with the use of GUS as a reporter gene in transgenic *Arabidopsis* plants.

To identify the subcellular compartment in which the new putative amino acid transporters are localized and understand the function of this proteins at a cellular level, the expression of GFP fusion proteins in *Arabidopsis* protoplasts shall be studied.

To know the possible role of these proteins in planta, a loss-of-function approach shall be followed. Isolation of T-DNA insertion lines and analysis of their phenotype shall help to this purpose.

2   Materials & Methods

2.1. Organisms

2.1.1. Bacterial strains

Escherichia coli DH5α: F⁻ *gyrA96* (*Nal^r*) *recA1 endA1 thi-1 hsdR17* (r_k^- m_k^+) *glnV44 deoR* Δ(lacZYA-argF)U169 [Φ80dΔ8(lacZ)*M15*]

Agrobacterium tumefaciens GV3101 (Koncz & Schell, 1986)

2.1.2. Yeast strains

The following *Saccharomyces cerevisiae* strains were used:

23344c: MATα *ura3-52* (Soussi-Boudekou *et al.*, 1997)

23346c: MATa *ura3-52* (Soussi-Boudekou *et al.*, 1997)

JT16: MATα *ura3-52 ino1 hip1-614 his4-401 can1* (Tanaka & Fink, 1985)

22Δ8AA: MATα *ura3-1 gap1-1 put4-1 uga4-1* Δcan1 Δapl1 Δlyp1 Δhip1 Δdip5 (Fischer *et al.*, 2002)

22Δ9AA: MATα *ura3-1 gap1-1 put4-1 uga4-1* Δcan1 Δapl1 Δlyp1 Δhip1 Δdip5 Δagp1 (section 2.1.2.1)

G119: MATα *ura3-52* Δavt1 Δasp1 Δleu2 pep4::nat (+ plasmid: YCPlac-V/Asp) (section 2.1.2.2)

2.1.2.1 Development of the 22Δ9AA strain

The 22Δ8AA strain was used to generate a mutant with an additional deletion. The coding sequence of *AGP1* was deleted by a disruption cassette containing *loxP* sites flanking *kan*. The mutant was selected on YPD containing 200 mg/l G418: the deletion was confirmed by PCR. The kan cassette was then removed as described (Güldener *et al.*, 1996).

2.1.2.2 Development of the G119 strain

The yeast strain 23344c was used to generate the G119 strain. The *AVT1, ASP1* and *LEU2* coding sequences were deleted by a disruption cassette containing *loxP* sites flanking *kan*, the *PEP4* gene by a *nat* cassette and each time the cassette was removed as described (Güldener *et al.*, 1996). The strain was then transformed with the YCPlac-V/Asp plasmid (section 2.5.4) and selection was made on minimal medium lacking leucine.

2.1.3. Plants

Arabidopsis thaliana L. Heynh, ecotype Columbia 0 (Col-0) was used for plant studies.

2.2 Vectors

Maps of the vectors used are shown in the appendices.

2.2.1 Bacterial vectors

pBS-110-GFP (Guillaume Pilot, unpublished)

pBS-111-GFP (Guillaume Pilot, unpublished)

pBS-173-35S (Guillaume Pilot, unpublished)

pCR4Blunt-Topo (Invitrogen)

2.2.2. Yeast expression vectors

pDR195 (Rentsch *et al.*, 1995)

pDR197 (Doris Rentsch, unpublished)

YCPlac111 (Gietz & Sugino, 1988)

pBHy11 (Horazdovsky *et al.*, 1994)

2.2.3. Binary vectors

pCB308 (Xiang *et al.*, 1999)

2.3. Media and cultures

2.3.1. Growth conditions

The bacteria were maintained, cultured, stored and transformed according to standard methods (Sambrook *et al.*, 2001). Yeast and bacterial cultures were grown in climatized growth chambers, *A. tumefaciens* and *S. cerevisiae* cultures at 28°C, *E. coli* at 37°C.

With the exception of young seedlings for histochemical analysis of the GUS activity, that were obtained by germinating the seeds on MS agar plates, all the other plants were grown in soil in the greenhouse. In the greenhouse, Arabidopsis was grown at a temperature of 18°C during the light period and 16°C during the dark period with a relative humidity of 50%. Light conditions averaged 100 μE, at 14 hours day and at 10 hours night, artificial illumination was used during winter times. In the growth chamber *Arabidopsis* plants were

grown at 22°C during the light period (16 hours) and 19°C during the dark period (8 hours). Light intensity averaged 100 μE, the relative humidity was 50%.

2.3.2. Media for bacterial cultures

E. coli was grown in Luria Broth (LB).

LB: 10 g l^{-1} tryptone; 5 g l^{-1} yeast extract; 10 g l^{-1} NaCl (Sambrook *et al.*, 2001)

A. tumefaciens was grown in YEB medium.

YEB: 5 g l^{-1} sucrose, 5 g l^{-1} peptone, 5 g l^{-1} beef extract, 1 g l^{-1} yeast extract,
      0.5 g MgSO$_4 \cdot$7H$_2$O; pH 7.2

Solid media were prepared adding 15 g agar per liter.

2.3.3. Media for yeast cultures

Saccharomyces cerevisiae was grown in either full medium (without plasmids) or in minimal medium lacking uracil (after transformation):

23344c strain and derivatives were grown on YPD medium. Transformants were selected and maintained on SD medium.

22Δ8AA strain and derivatives were grown on YPD medium. Transformants were selected and maintained on SD medium. Complementation tests were performed with BA medium supplemented with the appropriate amino acid as the nitrogen source.

JT16 strain and derivatives were grown on YPD medium supplemented with 600 μM histidine (Tanaka & Fink, 1985). Transformants were selected and maintained in SC (full His) and complementation tests were performed on HLM or SC (limited His).

YPD: 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose (Adams *et al.*, 1997)

SD: DOB + 0.77 g l^{-1} CSM-Ura

SC (full His): DOB + 30 mM His, 1.8 g l^{-1} SC-His-Ura

SC (limited His): DOB + 6 mM or 0.6 mM His, 1.8 g l^{-1} SC-His-Ura

HLM: DOB + 130 μM His, 0.002% inositol, 0.1%Arg (Hsu *et al.*, 1993)

DOB: 1.7 g l^{-1} YNB, 5 g l^{-1} (NH$_4$)$_2$SO$_4$, 20 g l^{-1} glucose

BA: Basic Medium + 20 g l^{-1} glucose, 0.1% BA microelements, 0,1% BA vitamins

Basic Medium: 0.8 mM MgSO$_4$, 5.7 mM KH$_2$PO$_4$, 2.7 mM CaCl$_2$, 8.6 mM NaCl, 5,7 mM K$_2$SO$_4$, 50 mM citric acid, 160 mM KOH, pH 6.1

Solid media were prepared adding 15 to 20 g agar per liter.

See the appendices for the formulation of YNB, CSM-Ura, SC-His-Ura, BA microelements and BA vitamins.

2.4. Cloning and sequencing

Standard methods such as plasmid preparations, digestions, electrophoresis, ligations, amplifications by PCR were performed as described (Ausubel *et al.*, 1996). The sequencing was done using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Warrington, UK) on an ABI Prism Sequencer 310 (Perkin Elmer, Warrington, UK).

2.5. Constructs

2.5.1. Cloning of ATs, ScAVT1 and AtANT1 cDNAs in yeast expression vector

The coding sequence (from ATG to the stop codon) of AT4, AT7, AT11 and AT12 was amplified by PCR from an *Arabidopsis thaliana* (Col-0) cDNA library in lambda phages. The

coding sequence of ScAVT1 was amplified by PCR from genomic DNA extracted from Saccharomyces cerevisiae, as the gene is intronless. The primers used were designed adding restriction sites on each, for cloning purposes (primers AT4F, AT4R for AT4; AT7F, AT7R for AT7; AT11F, AT11R for AT11; AT12F, AT12R for AT12; AVT1F and AVT1R for ScAVT1; sequences in section 6.3). The PCR product was digested with the appropriate restriction enzymes and ligated into pDR197 *Bam* HI / *Eco* RI (AT4, AT12), pDR197 *Pst* I / *Sal* I (AT7) or pDR195 *Bam* HI / *Xho* I (AT11, ScAVT1). The coding sequence of AtANT1 was taken from EST clone 42E3 (Arabidopsis Biological Resource center) and cloned by restriction in pDR197 digested with *Bam* HI and *Pst* I. All the inserts were checked by sequencing. pDR-AtAAP6 and pDR-AtCAT1 were available.
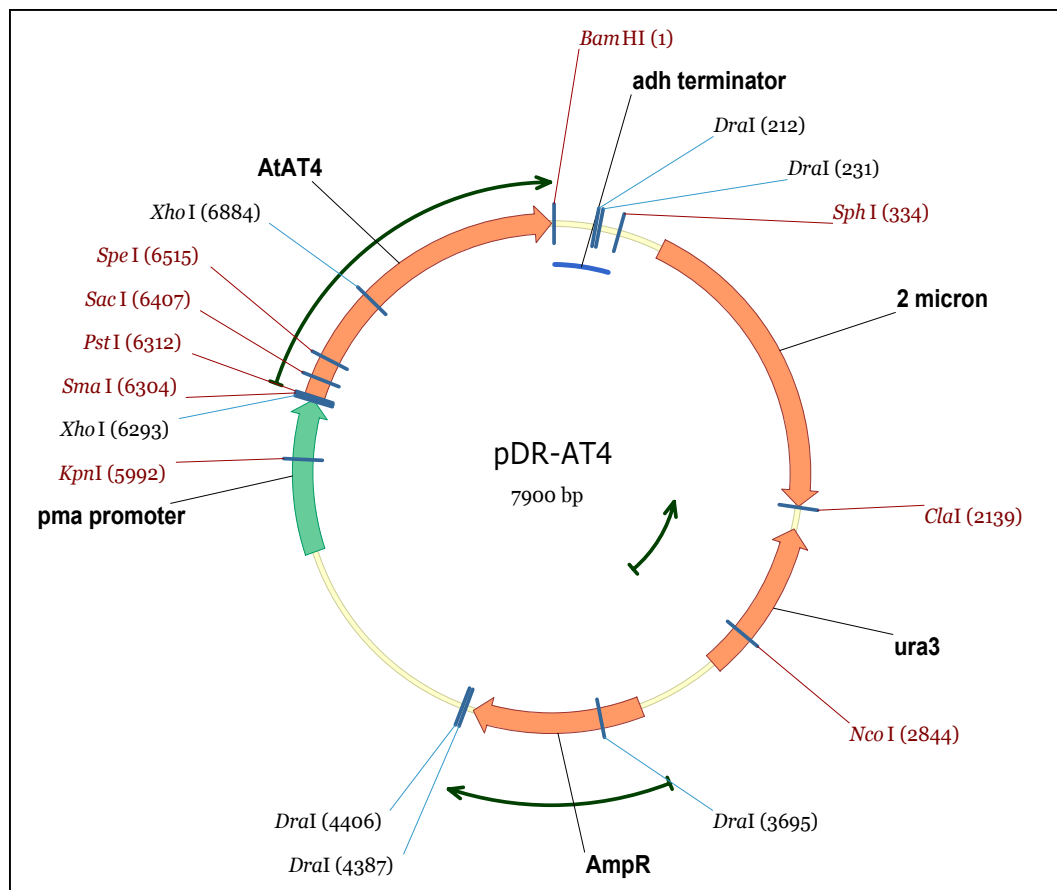
Figure 1 – Map of the pDR-AT4 construct
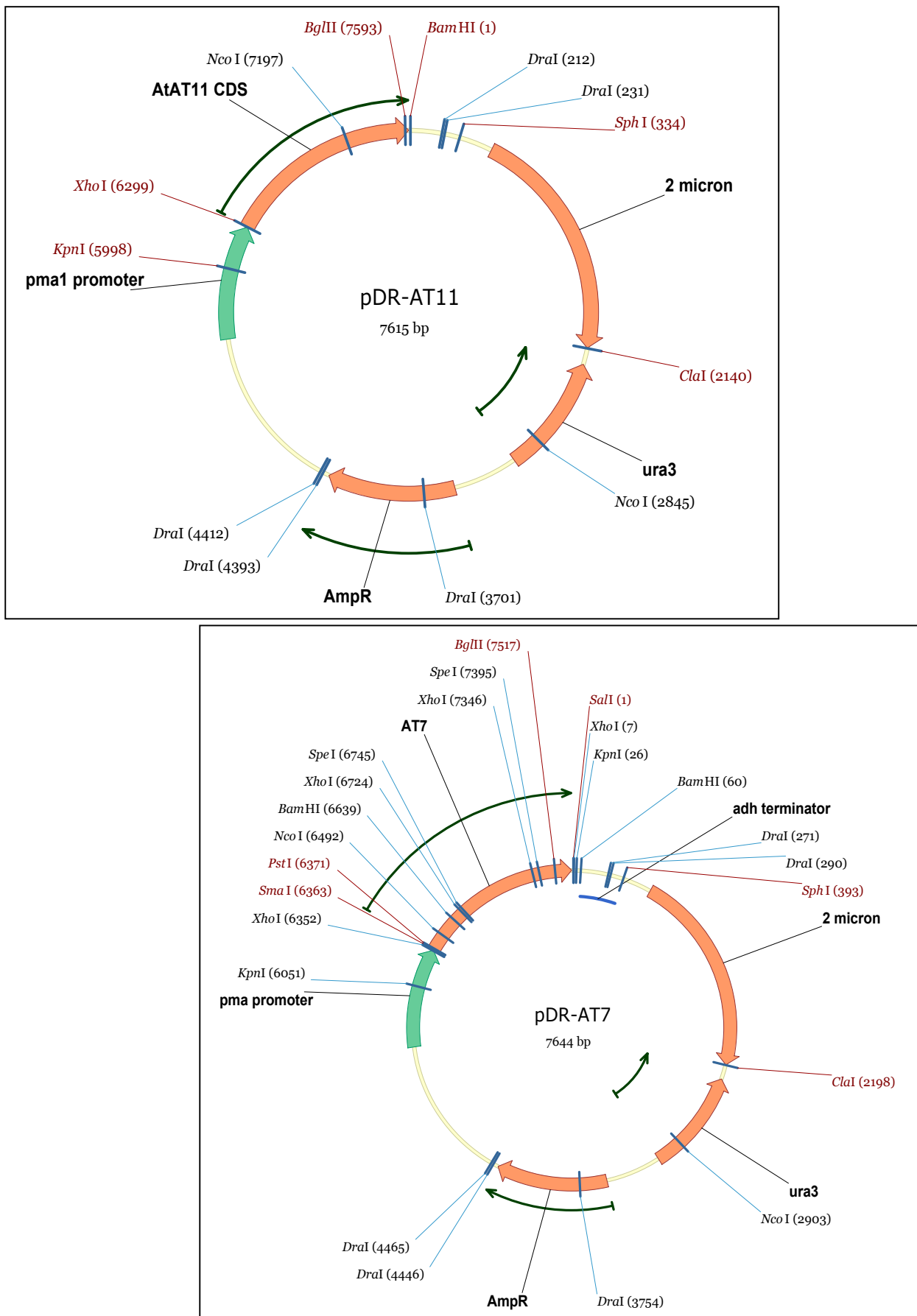
Figure 2 – Map of the pDR-AT7 (top) and pDR-AT11 (bottom) constructs
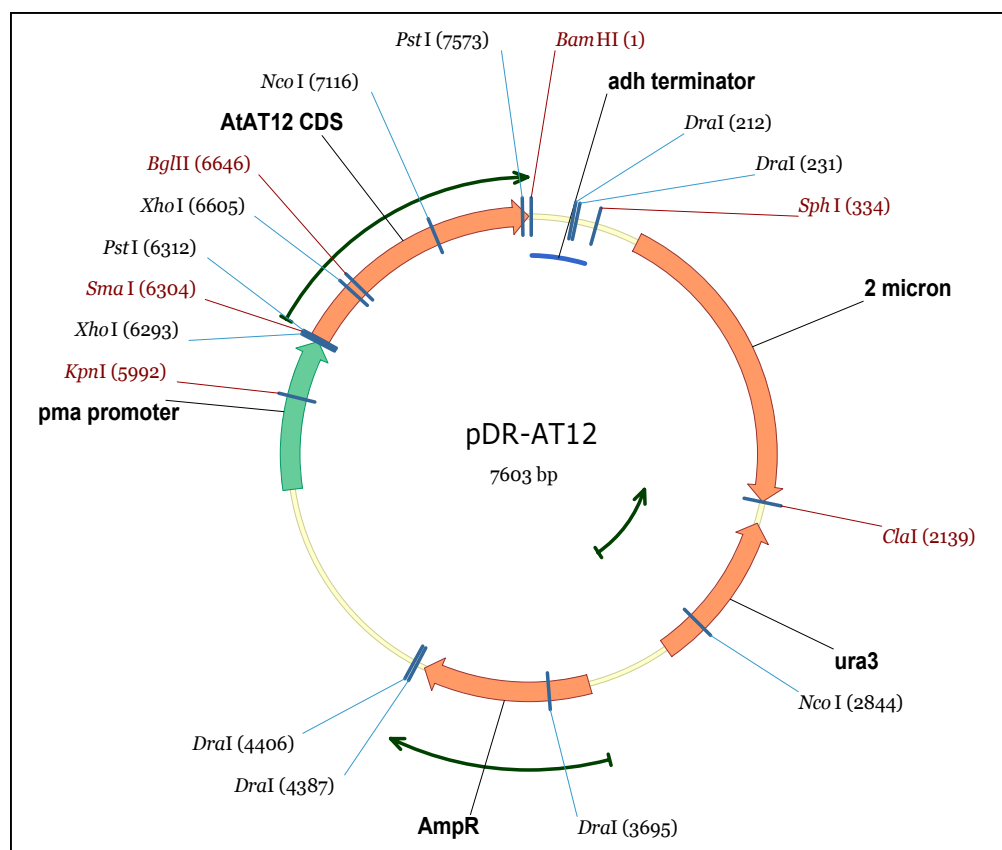
Figure 3 – Map of the pDR-AT12 construct

2.5.2. Cloning of ATs promoters in plant binary vector

The region between the end of the previous gene on the chromosome and the ATG of the AT gene was selected as a putative maximal promoter of the AT gene for AT4, AT5 and AT12. A region of 1973 bp upstream of AT9 was selected as putative maximal promoter of the gene AT9.

promoter	start	end	extension
AT4	end of 3'UTR of previous gene	ATG of AT4	604 bp
AT5	start of previous gene	ATG of AT5	1511 bp
AT9	2 kb upstream of AT9	ATG of AT9	1973 bp
AT12	stop codon of previous gene	ATG of AT12	722 bp

Table 1 – Regions selected as possible promoters of the AT genes.
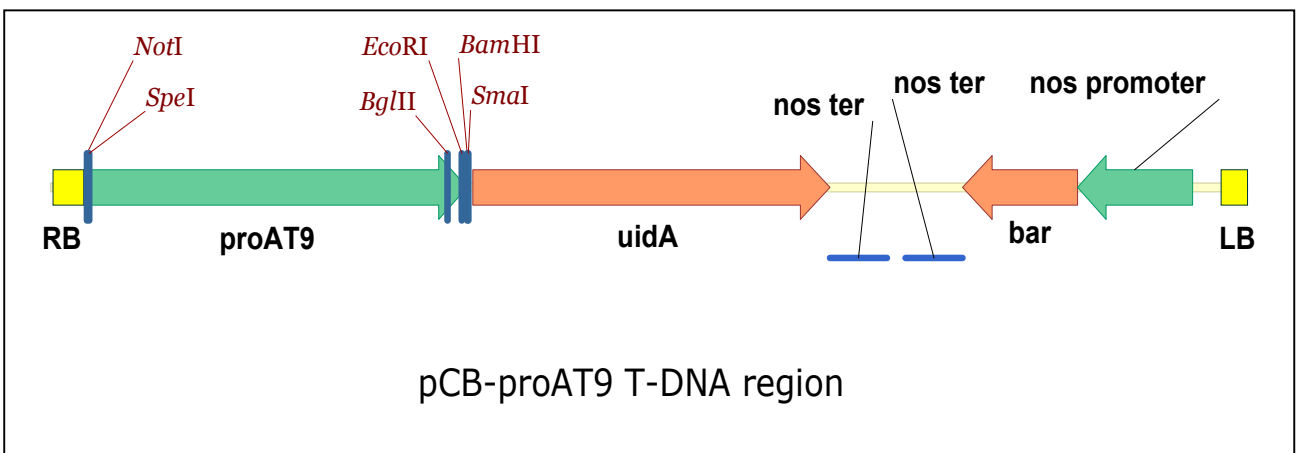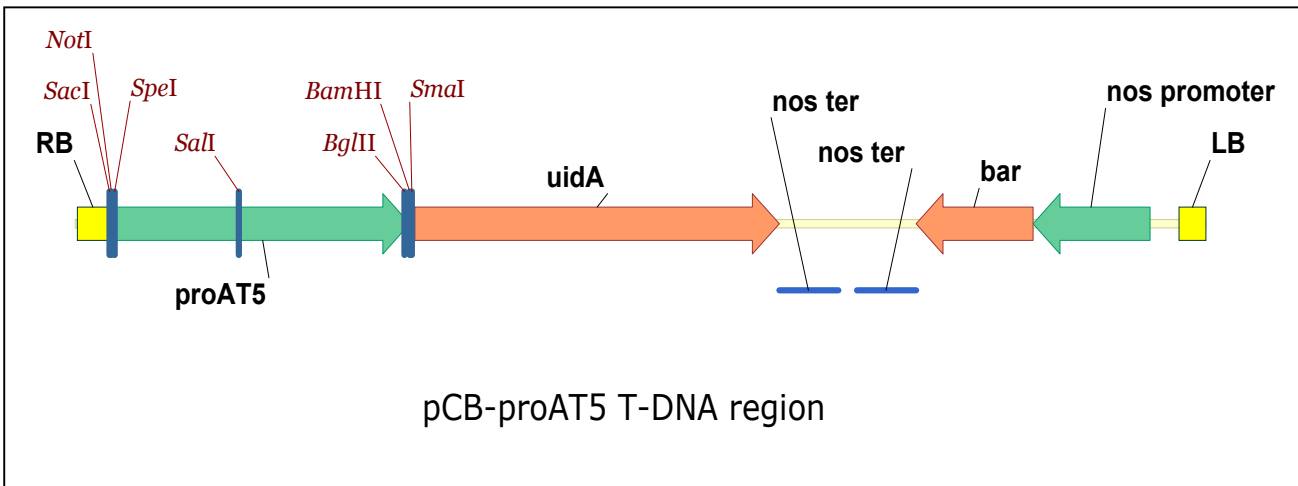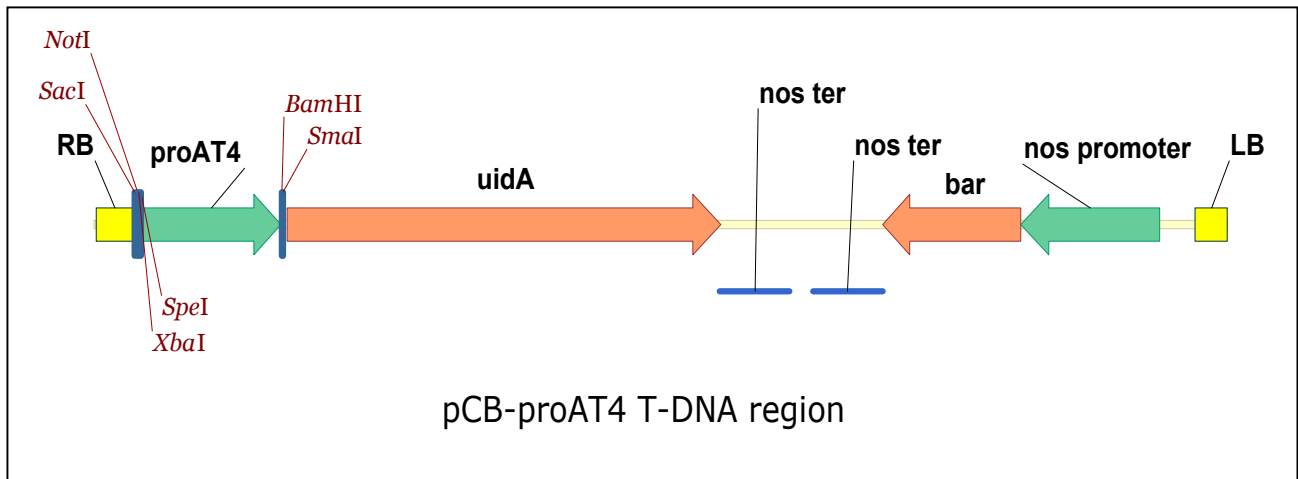
Figure 4 – Map of the pCB-proAT4, pCB-proAT5 and pCB-proAT9 constructs.
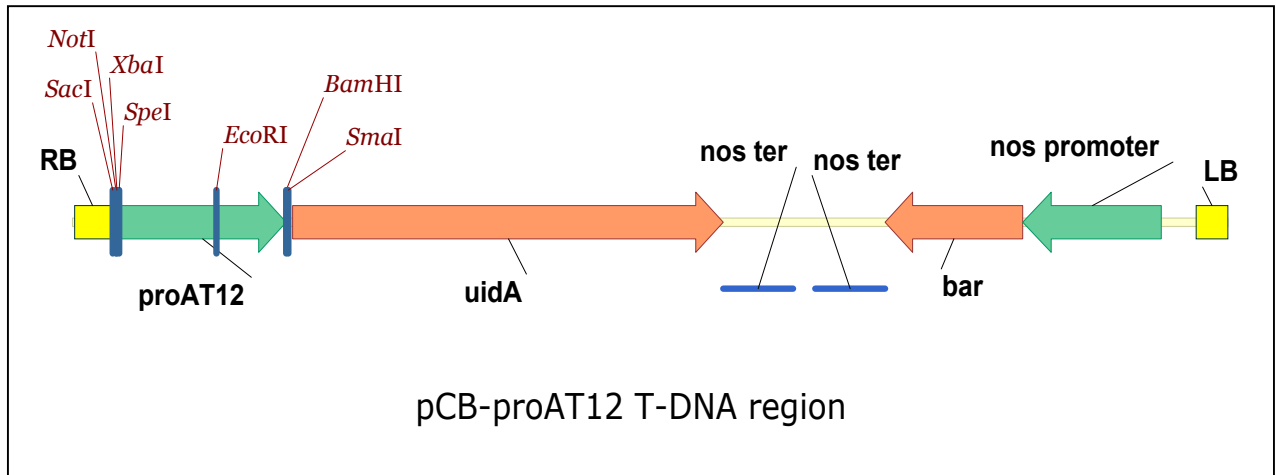
Figure 5 – Map of the pCB-proAT12 construct.

The selected region was amplified from genomic DNA extracted from *Arabidopsis thaliana* with appropriate primers (section 6.3) and cloned in the Topo vector pCR4-Blunt with the Topo cloning kit (Invitrogen). The identity of the inserts was confirmed by sequencing. They were then excised from the Topo vector and inserted in the *Bam* HI / *Spe* I sites in the polylinker of the T-DNA region of vector pCB-308.

2.5.3. Fusions of AT1, AT11 and AT12 with GFP and cloning into plant protoplast transformation vector

The AT12 coding sequence without the stop codon was amplified from the pDR-AT12 vector with appropriate primers containing an *Xba* I site in the F primer and an *Sal* I site in the R primer. The PCR product was cloned in the *Xba* I / *Sal* I sites in the polylinker of pBS-111-GFP (Guillaume Pilot, unpublished) in frame with the mGFP5 coding sequence. The AT12-GFP fusion coding sequence was checked by sequencing, then excised and inserted in the *Sac* I site of pBS-173-35S (Guillaume Pilot, unpublished), between the 35S promoter and the RBSC terminator.

The AT1 coding sequence was amplified from the flEST provided by Riken Consortium (pda10450) with appropriate primers containing a *Bam HI* site in the F primer and *Pst I* and *Spe I* sites in the R primer. The AT11 coding sequence was amplified from the pDR-AT11 vector with appropriate primers containing a *Bam HI* site in the F primer and *Pst I* and *Spe I* sites in the R primer. The PCR products were then cloned in the *Bam HI* / *Spe I* sites of pBS-

110-GFP (Guillaume Pilot, unpublished) in frame with the mGFP coding sequence, at the 3'
of it. The GFP-AT1 and GFP-AT11 fusions were checked by sequencing, then excised and
inserted in *Kpn I / Pst I* sites in pBS-173-35S (Guillaume Pilot, unpublished).
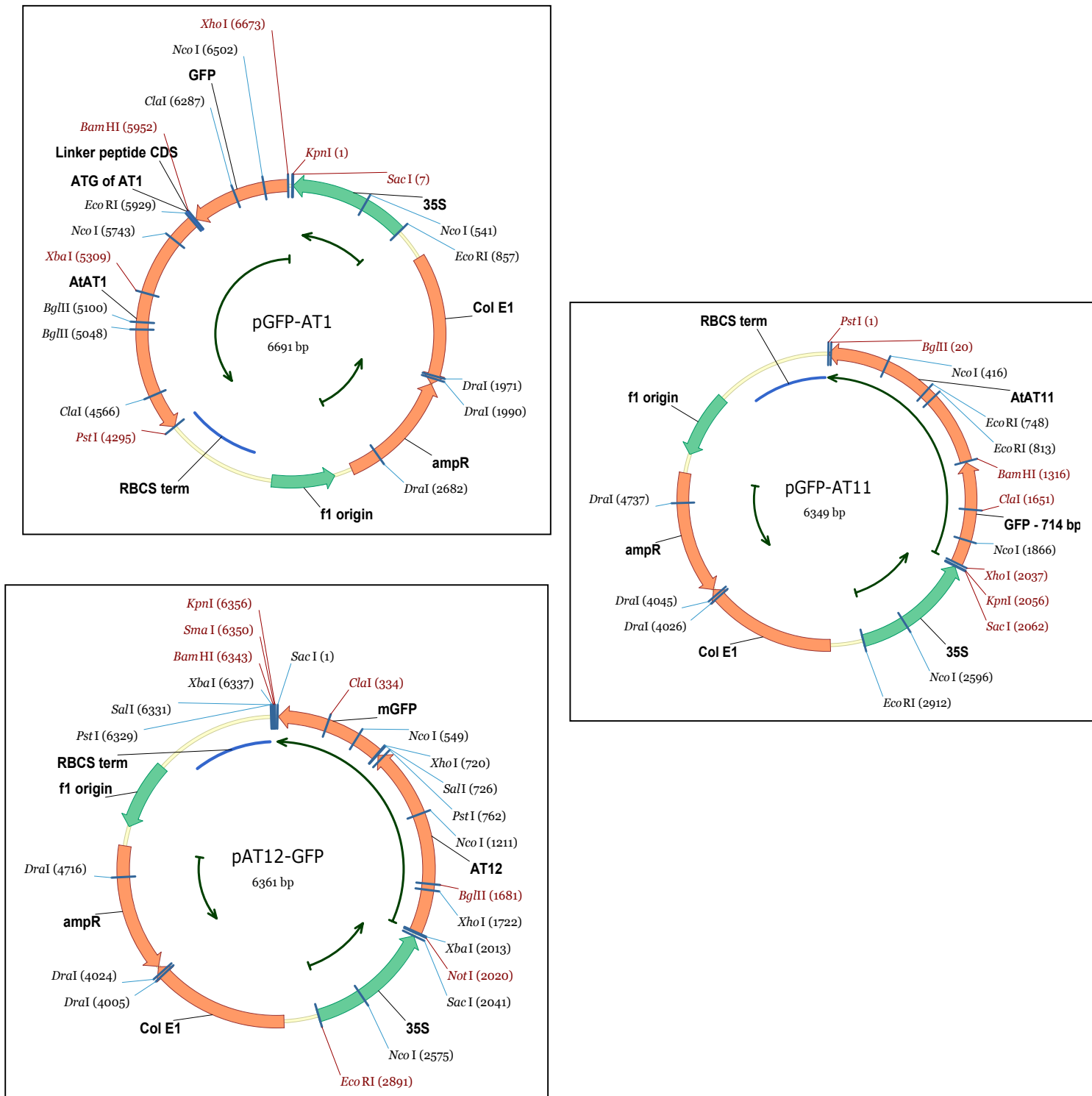
Figure 6 – Map of the pGFP-AT1, pGFP-AT11 and pAT12-GFP constructs

2.5.4. Construction of YCPlac-V/Asp

The Asp3 CDS was amplified by PCR from yeast genomic DNA with primers containing respectively *Not* I and *Bam* HI restriction sites, digested with these restriction enzymes and ligated into pBHy11 (Horazdovsky *et al.*, 1994). The resulting plasmid was named pBHy11-Asp3. The CPY promoter / leader sequence / Asp3 portion of pBHy11-Asp3 was cut with *Sac* I and *Sal* I (partial digestion). The larger (3 kb) digestion product was then cloned in *Sal* I and *Sac* I in YCPlac111 (Gietz & Sugino, 1988) to yield YCPlac-V/Asp.

2.6. Transformation

2.6.1. Bacterial transformation

Escherichia coli was transformed as described in Ausubel *et al.*, 1996.
Agrobacterium tumefaciens was transformed using the freeze-thaw method (Chen *et al.*, 1994). Competent cells were prepared as follows: 500 ml of culture at $OD_{600} = 0.5$ were harvested by centrifugation, washed with 100 ml of 0.15M NaCl and resuspended in 10 ml of ice-cold $CaCl_2$, then 200 μl aliquots were frozen in liquid nitrogen.
For the transformation the cells were thawed and incubated 5 minutes on ice with about 1 μg of plasmid DNA. The cells were then frozen again in liquid nitrogen, thawed at 37°C for 5 minutes, then incubated with 1 ml of YEB medium for 2 hours. Transformants were selected on YEB plates containing appropriate antibiotics (100 mg l^{-1} rifampicin, 25 mg l^{-1} gentamycin and 100 mg l^{-1} ampicillin).

2.6.2 Yeast transformation

Saccharomyces cerevisiae was transformed with the LiAc/ssDNA/PEG method (Gietz & Woods, 2002) and transformants were selected on minimal medium without uracil.

2.6.3 Plant transformation

Arabidopsis thaliana plants were transformed by floral dipping (Clough & Bent, 1998) and transgenic plants were selected for BASTA resistance.

2.6.4. Protoplast transformation

Arabidopsis thaliana protoplasts were transformed using the PEG mediated transformation according to the procedure described for tobacco protoplasts (Negrutiu *et al.*, 1987).

2.7. Yeast uptake assays

2.7.1. Isolation of vacuolar vesicles and uptake assay

Vacuolar vesicles derived from the yeast vacuole were isolated by preparation of spheroplasts, homogenization with Potter homogenizer and ultracentrifugation, as described by Roberts *et al.* (1991). The vesicles prepared by this method are spherical, with a diameter of 0.2 to 1.6 µM, in right-side-out orientation (Uchida *et al.*, 1988). The protein concentration was measured with the amido-black method (Kaplan & Pedersen, 1985). The uptake assay was performed with vesicles corresponding to 10 µg protein resuspended in a final volume of 310 µl in the vacuolar uptake buffer (20 mM MES-Tris pH 6.9, 4 mM MgSO4 and 20 mM KCl), energized incubating with 110 µl of 6 mM ATP for 1 min, then incubated with 20 µl of substrate containing the radioactive substrate (37 KBq for Gln; 3,7 KBq for Lys) and the non-radioactive substrate to the desired concentration. 100 µl aliquots were collected after 30, 60, 120 and 180 sec. The vesicles were collected on a glass fiber filter by vacuum filtration and the filter was washed twice with 4 ml ice-cold buffer. The filter was then transferred in 5 ml scintillation cocktail and the radioactivity measured with a scintillation counter.

2.7.2. Uptake assay on whole yeast cells

The cells of an overnight yeast culture at $OD_{600} = 0,5$ were harvested by centrifugation, washed with water, washed twice with uptake buffer (100 mM potassium phosphate pH 4.5) and resuspended in the same buffer at $OD_{600} = 5.0$ exactly. 100 µl of the cells so prepared were energized incubating with 10 µl 1M Glc for 5 minutes, then incubated with the 110 µl of substrate (1,85 KBq radioactive substrate + non-radioactive substrate to the desired concentration) and dissolved in the uptake buffer. 50 µl aliquots were collected after 60, 120, 180 and 300 sec. The cells were collected on a glass fiber filter by vacuum filtration and the filter was washed twice with 4 ml ice cold uptake buffer. Then the filter was put in 5 ml scintillation cocktail and the radioactivity measured with a scintillation counter.

2.8. Yeast mutants complementation assays

For the complementation assays of the JT16 yeast strain and of the 22Δ9AA strain yeast liquid cultures were grown to an OD of 0.8-1.0. To calculate the approximate number of cells contained in the cultures, each OD unit was considered to contain 3×10^7 cells; serial dilutions of the culture were prepared to a concentration of 10^5, 10^4, 10^3, 10^2 and 10 cells for each 20 μl of culture. 20 μl of each dilution were then pipetted in spots at an approximate distance of 1 cm each from each other on appropriate plates.

2.9. GUS staining

The histochemical staining of the GUS activity in transgenic plants expressing the GUS gene under control of the ATs promoters was performed as described in Martin *et al.*, 1992.

2.10 Bioinformatic methods

Similarity searches were performed using the BLAST (Basic Local Alignment Search Tool) tool at http://www.ncbi.nlm.nih.gov/blast/ or through the Aramemnon database at http://crombec.botanik.uni-koeln.de/ . Transmembrane domain predictions were performed using TMHMM Server, v.2.0 at http://www.cbs.dtu.dk/services/TMHMM/ . Intracellular targeting predictions were taken from the Aramemnon database at http://crombec.botanik.uni-koeln.de/ .
The analysis of interpro domains was taken from the TAIR database (http://www.arabidopsis.org). The alignment of the ATF1 family proteins and the relative phylogenetic tree (figure 7) was constructed using the alignment tool ClustalW (http://www.ebi.ac.uk/clustalw/) and AlignX of Vector NTI Suite 7.0 (InforMax, Inc.). T-DNA insertions lines and ESTs were searched for using Signal Salk database (http://signal.salk.edu/).

2.11. Confocal fluorescent microscopy

Protoplasts transformed with the GFP-AT1, GFP-AT11 and AT12-GFP constructs were incubated for 24, 48 or 72 hours at 24°C in the dark and observed with a Leica DM RE confocal microscope (Leica, Germany).

3 Results

3.1. *In silico* identification of new *A. thaliana* amino acid transporters

3.1.1. Sequence similarities reveal new subfamilies of putative amino acid transporters

To better understand the complex picture of amino acid transport in plants, information about the complete set of proteins involved is required. In this context, sequence similarity and the definition of evolutionary related protein families and subfamilies have been a powerful tool for the identification of novel transporters and in particular for the fully sequenced genome of the model plant *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000).

The AVTs represent a family of yeast proteins whose function was first determined as amino acids transporters at the tonoplast (Russnak *et al.*, 2001). However more recently another study has indicated the AVTs as transporters of auxin and it has not confirmed the tonoplast localization (Prusty *et al.*, 2004).

To identify proteins related to the ScAVTs in *Arabidopsis thaliana*, a BLAST search was performed, using the protein sequence of ScAVT1 as query, in the Aramemnon database of *Arabidopsis* and rice membrane proteins (Schwacke *et al.*, 2003). The result is shown in Table 2.

The proteins whose sequences are more related to the yeast AVT1 are the 10 putative amino acid transporters AT1 to 10, which do not share similarities with known transporters, followed by AtANT1, which was demonstrated to be a transporter for aromatic and neutral amino acids in *Arabidopsis* (Chen et al., 2001).

Another BLAST search was then performed, looking for homologies to ScAVT3, using the same search parameters but the amino acid sequence of ScAVT3 as query. The results obtained are shown in Table 3. The best homology scores are found with three other putative amino acid transporters (AT11 to AT13) and AtANT1.

A third BLAST search, looking for homologies to ScAVT6, using the same search parameters but the amino acid sequence of ScAVT6 completed the structured examination of the putative amino acid transporters present in the AAAP family. The results are shown in Table 4.

```
BLASTP 2.2

Database: Aramemnon 2.0 - 22 April 2004

Limited to: Arabidopsis thaliana membrane proteins

Query: Protein sequence of ScAVT1
```

Gene code	Gene name	Score	E-value	Subfamily
At5g02170	AT4	172	2e-43	ATs / ANT1 / ScAVT1
At2g39130	AT9	160	7e-40	
At5g02180	AT1	155	1e-38	
At3g09340	AT2	144	4e-35	
At3g09330	AT3	143	7e-35	
At3g54830	AT8	142	1e-34	
At3g28960	AT6	135	2e-32	
At5g15240	AT7	134	3e-32	
At2g41190	AT5	132	2e-31	
At5g16740	AT10	110	5e-25	
At3g11900	AtANT1	89	3e-18	
At3g30390	AVT2,5,6,7 related 4	54	5e-08	AVT2-5-6-7
At5g41800	ProT related 1	53	1e-07	ProTs

Table 2 – Results of BLAST searches on entries of the Aramemnon database (Schwacke *et al.*, 2003) using ScAVT1 as query sequence. The AT genes from AT1 to AT10 are the most related homologues of ScAVT1 in Arabidopsis.

```
BLASTP 2.2

Database: Aramemnon 2.0 - 22 April 2004

Limited to: Arabidopsis thaliana membrane proteins

Query: Protein sequence of ScAVT3
```

Gene code	Gene name	Score	E-value	Subfamily
At5g65990	AT12	132	2e-31	AT11-13 / ANT1 / ScAVT1
At4g38250	AT11	120	7e-28	
At2g42005	AT13	115	2e-26	
At3g11900	ANT1	106	1e-23	
At1g10010	AAP8	71	6e-13	AAPs
At2g40420	AVT2,5,6,7 related 2	65	5e-11	ScAVT2-5-6-7
At1g25530	LHT related 2	62	2e-10	LHTs
At5g02180	AT1	61	5e-10	AT1-10 / ScAVT3-4
At3g56200	AVT2,5,6,7 related 3	60	1e-09	ScAVT2-5-6-7
At3g09330	AT3	57	1e-08	AT1-10 / ScAVT3-4
At3g01760	LHT6	56	2e-08	LHTs
At3g09340	AT2	56	2e-08	AT1-10 / ScAVT3-4
At1g58360	AAP1	55	3e-08	AAPs
At3g30390	AVT2,5,6,7 related 4	55	4e-08	ScAVT2-5-6-7
At5g02170	AT4	54	8e-08	AT1-10 / ScAVT3-4
At2g41190	AT5	54	1e-07	
At5g40780	LHT1	54	1e-07	LHTs
At5g49630	AAP6	53	1e-07	AAPs
At1g71680	LHT8	51	7e-07	LHTs

Table 3 – Results of BLAST searches on entries of the Aramemnon database (Schwacke *et al.*, 2003) using ScAVT3 as query sequence. The AT genes from AT11 to AT13 and AtANT1 are the most related homologues of ScAVT3 in Arabidopsis.

```
BLASTP 2.2

Database: Aramemnon 2.0 - 22 April 2004

Limited to: Arabidopsis thaliana membrane proteins

Query: Protein sequence of ScAVT6
```

Gene code	Gene name	Score	E-value	Subfamily
At1g80510	AVT2,5,6,7 related 1	100	8e-22	ScAVT2-5-6-7
At5g38820	AVT2,5,6,7 related 5	99	2e-21	
At3g30390	AVT2,5,6,7 related 4	93	9e-20	
At3g56200	AVT2,5,6,7 related 3	84	3e-17	
At2g40420	AVT2,5,6,7 related 2	82	1e-16	
At5g02180	AT1	51	4e-07	AT1-10 / ScAVT3-4
At3g54830	AT8	50	7e-07	

Table 4 – Results of BLAST searches on entries of the Aramemnon database (Schwacke *et al.*, 2003) using ScAVT6 as query sequence. Five putative amino acid transporters (At1g80510; At2g40420; At3g30390; At3g56200; At5g38820) are the closest relatives to ScAVT6.

A phylogenetic tree with all known transporters of *Arabidopsis thaliana* and *Saccharomyces cerevisiae* belonging to the AAAP family and their related proteins was then constructed (Fig.7). All transmembrane proteins related to one or more functionally characterized transporters were included.

In the AAAP family (amino acid-auxin permease), alias ATF1 family (amino acid transporters family), the proteins whose function have been determined are transporters of amino acid, i.e. AtAAPs (Fischer *et al.*, 1995), AtLHTs (Chen & Bush, 1997), AtProT1 and AtProT2 (Breitkreuz *et al.*, 1999, Rentsch *et al.*, 1996), AtANT1 (Chen *et al.*, 2001) or auxin, i.e. AtAux1 (Bennett *et al.*, 1996). The yeast ScAVTs have been reported as vacuolar transporters of amino acids (Russnak *et al.*, 2001) or auxin. Some amino acid transporter of other organisms, like RnVGAT from rat (McIntire *et al.*, 1997) belong also to the same family.

Several members of the AAAP family have not been functionally characterized. AtProT1 and AtProT2 (Breitkreuz *et al.*, 1999, Rentsch *et al.*, 1996), one close related protein (AtProT3) and two other homologs (At1g08230 and At5g41800) belong to the ProT family. AtLHT1

(Chen & Bush, 1997) is a member of a family consisting of 10 amino acid transporters (AtLHT1-8, At1g25530 and At1g48640). Eight AAPs are known and they have all been functionally characterized with the exception of AtAAP7 (Okumoto *et al.*, 2002). AtAUX1 (Bennett *et al.*, 1996) belongs to a small family consisting of 4 amino acid transporters (AUX1, At1g77690, At2g21050 and At5g02140).

The other members are not closely related to AAPs, LHTs, ProTs or Aux1. These proteins belong to three families. Ten proteins are most related to ScAVT1 (AT1 to 10), three others are closely related to ScAVT3 and 4 and AtANT1 (AT11 to 13). Five other proteins are closely related to ScAVT2, 5, 6 and 7 (At1g80510; At2g40420; At3g30390; At3g56200; At5g38820).

The studies of this project focused on the proteins of subfamilies with undetermined function, which are related to ScAVT1 and to ScAVT3/ScAVT4/AtANT1. Selected features of these genes and their mRNAs and predicted proteins are summarized in Table 5 and 6.

	gene		mRNA	
Name	Gene code	Introns	ESTs	Present in whole genome array (Yamada *et al.*, 2003)
AT1	At5g02180	10	RAFL17-02-L16	Yes
AT2	At3g09340	9	no EST available	No
AT3	At3g09330	9	no EST available	No
AT4	At5g02170	10	RAFL09-34-B13	Yes
AT5	At2g41190	10	RAFL08-12-B05 RAFL05-09-P10 RAFL05-20-J01 GSLTLS11ZF11	Yes
AT6	At3g28960	2	no EST available	No
AT7	At5g15240	2	GSLTFB34ZA09	No
AT8	At3g54830	11	RAFL14-05-J18	No
AT9	At2g39130	9	GSLTLS24ZF10 RAFL07-10-M22	Yes
AT10	At5g16740	2	no EST available	Yes
AT11	At4g38250	0	RAFL05-18-C06 GSLTPGH48ZE07 GSLTFB64ZH03	Yes
AT12	At5g65990	1	RAFL05-16-E11 GSLTBX829565 GSLTBX830854	Yes
AT13	At2g42005	0	RAFL19-52-E05	No

Table 5 – Features of the AT genes and their mRNAs. From left to right columns: the name of the gene, the entry name according to the MIPS nomenclature, the number of introns present in the gene, the entry code of available ESTs and the verification of the transcription in the whole genome array by Yamada *et al.*, 2003. In the ESTs list the beginning of the name indicate to which collection the EST belong: RAFL = RIKEN Arabidopsis Full Lenght cDNA collection (Seki *et al.*, 2002), GSLT = Genoscope cDNA collection (Castelli *et al.*, 2004).
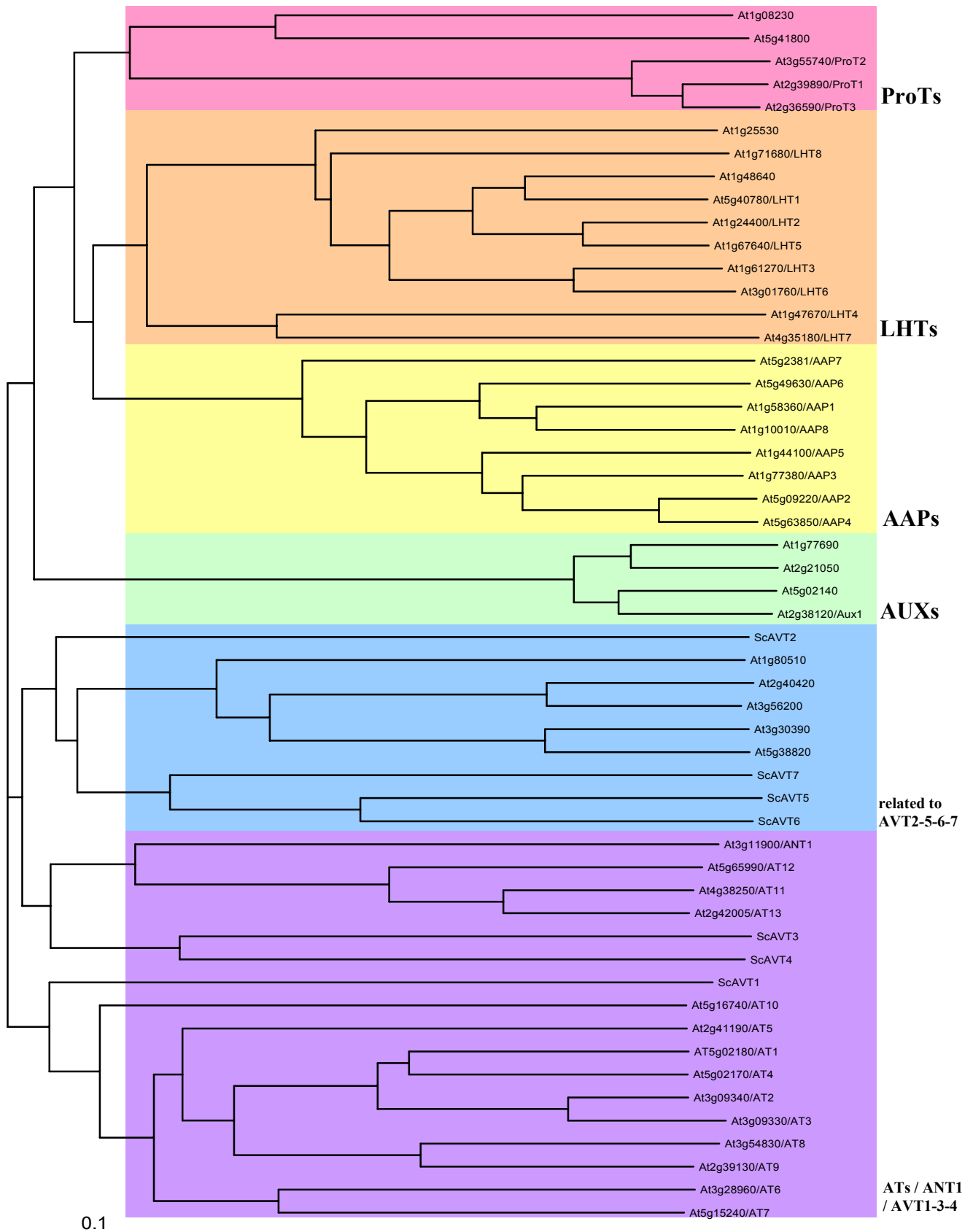
Figure 7 – Phylogenetic tree of the members of the AAAP superfamily (auxin-amino acid permeases) / ATF1 (Amino acid transporter family 1) in *Arabidopsis thaliana* and in *Saccharomyces cerevisiae*

The genes for AT1, AT4 and for AT2, AT3 are located in tandem on the chromosome with similar exons-introns structure, suggesting a possible origin by gene duplication. The genes AT1 to AT5, AT8 and AT9 have a high number of introns (9 to 11), while the others have few introns (AT6, AT7, AT10, AT12) or are intronless (AT11, AT13). The ORF of AT1 to AT12 were predicted from the genome sequence by gene prediction programs. AT13 was only recently added to this list, as its CDS was initially not predicted and the existence of the gene was deduced from a flEST from Riken Consortium only recently sequenced and made available.

For the genes AT1, AT4, AT5, AT9, AT11, AT12 and AT13 full length ESTs from Riken (Seki *et al.*, 2002) are available. A partial EST from Riken is also available for the AT8 gene. For AT5, AT7, AT9, AT11 and AT12 cDNAs from GSLT (Castelli *et al.*, 2004) are available (data from Signal SALK website at http://signal.salk.edu/; entry codes are reported in Table 5). No ESTs are available for AT2, AT3, AT6 and AT10. The transcription of the AT1, AT4, AT5, AT7, AT8, AT9, AT10, AT11 and AT12 genes has been verified by a whole genome array (Yamada *et al.*, 2003) (data from MATDB website at http://mips.gsf.de/proj/thal/db/). In conclusion, for AT2, AT3 and AT6 there is no evidence that the genes are expressed, while the annotation of the other genes is supported either by cDNA clones or the whole genome array.

Name	Gene code	Amino acids	Isoeletric point	TMDs (TMHMM2)	TMDs (Aramemnon)
AT1	At5g02180	550	5.00	9	10
AT2	At3g09340	478	4.73	9	11
AT3	At3g09330	481	5.42	7	11
AT4	At5g02170	526	6.10	11	11
AT5	At2g41190	536	4.76	11	10
AT6	At3g28960	361	8.91	9	10
AT7	At5g15240	423	7.98	10	11
AT8	At3g54830	571	4.81	6	8
AT9	At2g39130	550	5.48	9	9
AT10	At5g16740	426	9.00	11	11
AT11	At4g38250	437	6.22	10	10
AT12	At5g65990	427	7.04	11	11
AT13	At2g42005	413	7.99	9	11

Table 6 – In silico analysis of the predicted proteins of the AT genes. From left to right: the entry name according to MIPS nomenclature, the number of amino acids of which the protein is composed, the calculated isoeletric point of the protein (MIPS website), the predicted number of transmembrane domains using the program TMHMM version 2.0 and the average predicted number of transmembrane domains reported in Aramemnon.

To determine the intracellular localization of the proteins, the signal peptide prediction using different algorithms is available in the Aramemnon database. The results of some of these prediction programs are reported in Table 7. The results indicate a prediction for a possible chloroplast localization (more than 50% with ChloroP) for AT11 and AT12. A value higher than 30% is also present for AT6 and AT13 as chloroplast signal prediction with ChloroP and as secretory pathway signal prediction with SignalP_NN. However the values are not very high and in general it is appropriate to say that a clear localization to the chloroplasts or to the mitochondria or to the secretory pathway cannot be deduced from the predictions.
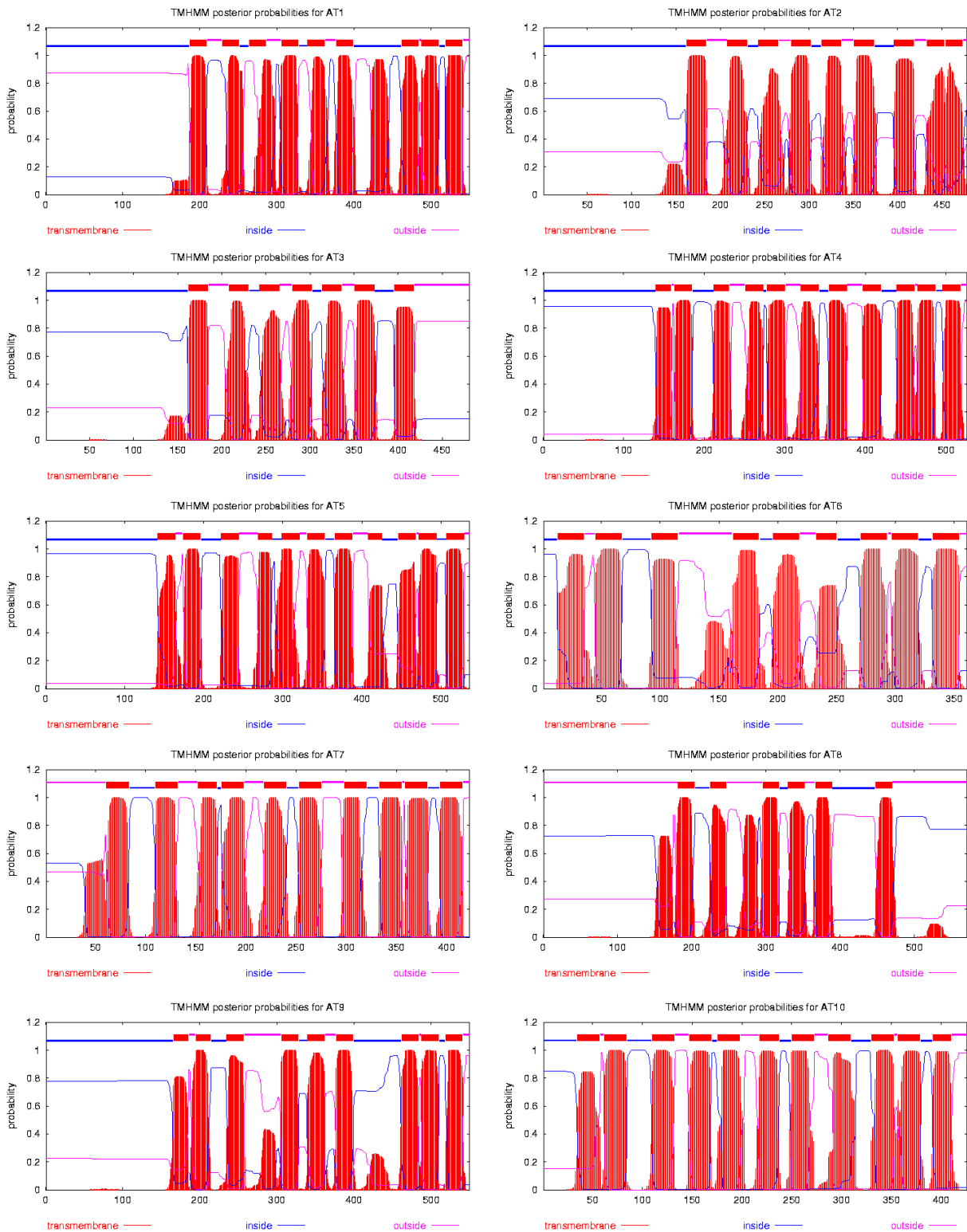
Name	Gene code	ChloroP (Chlor)	MitoProt (Mitoch)	SignalP_NN (Secr Path)	TargetP (Chlor)	TargetP (Mitoch)	TargetP (Secr Path)
AT1	At5g02180	0.05	0.01	0.06	0.03	0.09	0.02
AT2	At3g09340	0.23	0.01	0.05	0.07	0.05	0.01
AT3	At3g09330	0.23	0.01	0.04	0.06	0.04	0.01
AT4	At5g02170	0.26	0.01	0.04	0.10	0.03	0.01
AT5	At2g41190	0.11	0.00	0.05	0.06	0.09	0.04
AT6	At3g28960	0.39	0.04	0.38	0.08	0.16	0.03
AT7	At5g15240	0.19	0.03	0.06	0.02	0.08	0.06
AT8	At3g54830	0.20	0.00	0.04	0.05	0.06	0.01
AT9	At2g39130	0.14	0.00	0.05	0.03	0.09	0.02
AT10	At5g16740	0.16	0.00	0.15	0.07	0.04	0.06
AT11	At4g38250	0.55	0.03	0.19	0.16	0.02	0.07
AT12	At5g65990	0.62	0.03	0.15	0.17	0.12	0.03
AT13	At2g42005	0.30	0.02	0.41	0.04	0.32	0.02

Table 7 – Probabilities of the presence of a signal peptide in the AT proteins according to several prediction programs, as reported in Aramemnon. From left to right the prediction of ChloroP for chloroplast signal, of MitoProt for mitochodrial signal, of SignalP_NN for a secretory pathway signal, and the prediction of TargetP for chloroplastic, mitochondrial and secretory pathway signal. The prediction with a value higher than 30% are shaded.

3.1.2. Transmembrane domains and amino acid permease signatures suggest that ATs are amino acid transporters

The analysis of interpro domains predicted from the protein sequence (data from TAIR database at http://www.arabidopsis.org/) has revealed that all the ATs share a signature called AA_Rel_permease_2 for amino acid permeases (accession PF01490 and PS50286) and all except AT13 share a signature called Aromatic_AA_permease for aromatic amino acid permeases (accession TIGR00837). This strengthens the hypothesis that these proteins have a function as amino acid permeases and indicate possible substrates.

All the protein sequences of the ATs were submitted to TMHMM program for prediction of transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The results are shown in Figure 8. The analysis of the putative transmembrane domains (TMDs) shows that their number varies from 9 to 11, with the exception of AT8 (6) and AT3 (7). Aramemnon reports an average number of predicted transmembrane domains, on the basis of the prediction of 11 different softwares (Schwacke *et al.*, 2003). This value is in agreement with the TMHMM prediction in the case of AT4, AT9, AT10, AT11 and AT12 and differs slightly in the other cases, with a number of transmembrane domains between 8 and 11 (Table 6).

TMHMM posterior probabilities for AT1

TMHMM posterior probabilities for AT2

TMHMM posterior probabilities for AT3

TMHMM posterior probabilities for AT4

TMHMM posterior probabilities for AT5

TMHMM posterior probabilities for AT6

TMHMM posterior probabilities for AT7

TMHMM posterior probabilities for AT8

TMHMM posterior probabilities for AT9

TMHMM posterior probabilities for AT10

46

Figure 8 – Hydrophobicity profiles and transmembrane prediction by TMHMM v.2.0 of the 13 AT proteins.

Two common patterns appear in the analysis. Firstly, in the case of AT1, AT2, AT3, AT4, AT5, AT8 and AT9 there is a long cytoplasmic N-terminal domain of about 150 amino acids present, followed by 6 to 11 TMDs; secondly, in the case of AT6, AT7, AT10, AT11, AT12, AT13 the N terminal domain is short (about 20 amino acids), followed by 9 to 11 TMDs.

In conclusion, the presence of multiple transmembrane domains and the amino acid permease signatures suggest that the ATs function as amino acid permeases in *Arabidopsis thaliana*.

3.2. Heterologous expression of ATs in yeast

3.2.1. Cloning of AT4, AT7, AT11 and AT12 in yeast expression vector

Saccharomyces cerevisiae has been successfully used as heterologous system for structure-function analysis of membrane proteins from different organisms (Bill, 2001). To functionally characterize the AT proteins, the coding sequences for the ATs were cloned in yeast expression vectors, pDRs (Rentsch *et al.*, 1995), and the resulting constructs were introduced in yeast.

The approach followed was amplification by PCR from an Arabidopsis cDNA library and direct cloning in the yeast expression vectors, pDR195 or pDR197 (section 2.5.1.). This approach was successful for AT4, AT7, AT11 and AT12, which were therefore chosen for transport studies.

ScAVT1 and AtANT1 were cloned in the same vector as controls.

3.2.2 Generation of the 22Δ9AA strain and complementation assay with ATs

The 22Δ8AA strain (Fischer *et al.*, 2002) has deletions in eight of the genes coding for known amino acid transporters on the plasma membrane: GAP1 (general amino acid permease), CAN1 (arginine permease), HIP1 (histidine permease), PUT4 (proline permease), LYP1 (lysine permease), ALP1 (basic amino acid permease), UGA4 (GABA permease), DIP5 (glutamate and aspartate permease. A number of other permeases remains active, like GNP1 (glutamine permease), PAP1 (low affinity branched amino acid permease), MUP1 and MUP3 (methionine permeases), BAP2 (leucine, valine and isoleucine permease, TAT1 (tyrosine and tryptophane permease), AGP1 (amino acid general permease) (Regenberg *et al.*, 1999).

A precursor of this strain (22547d) was successfully used to isolate and characterize new amino acid transporters. The inability of this strain to grow on glutamate, aspartate, proline, arginine, GABA or citrulline as sole nitrogen source was used as a complementation system to characterize amino acid transporters.

To increase the sensitivity of the system, the gene for a further general amino acid permease, AGP1, was deleted by homologous recombination. The new strain was called 22Δ9AA. Yeasts of the 22Δ9AA strain transformed with the AT4, AT7, AT11 and AT12 constructs were cultured on minimal medium in which the source of nitrogen is either ammonium, glutamine (positive controls) or one of the amino acids mentioned before (glutamate, aspartate, proline, arginine, GABA, citrulline) on which the 22Δ9AA strain is not able to grow. The same yeast strain transformed with a construct expressing AtAAP6 was used as a positive control. While yeast expressing AtAAP6 was able to grow on all media except the one with glutamate as sole nitrogen source, the yeast expressing the four AT genes were not able to grow on any of the tested amino acids. None of the four genes was able to complement the system (Fig. 9). This indicates that none of these putative transporters is able to effectively transport these amino acids under the tested conditions and complement this strain.
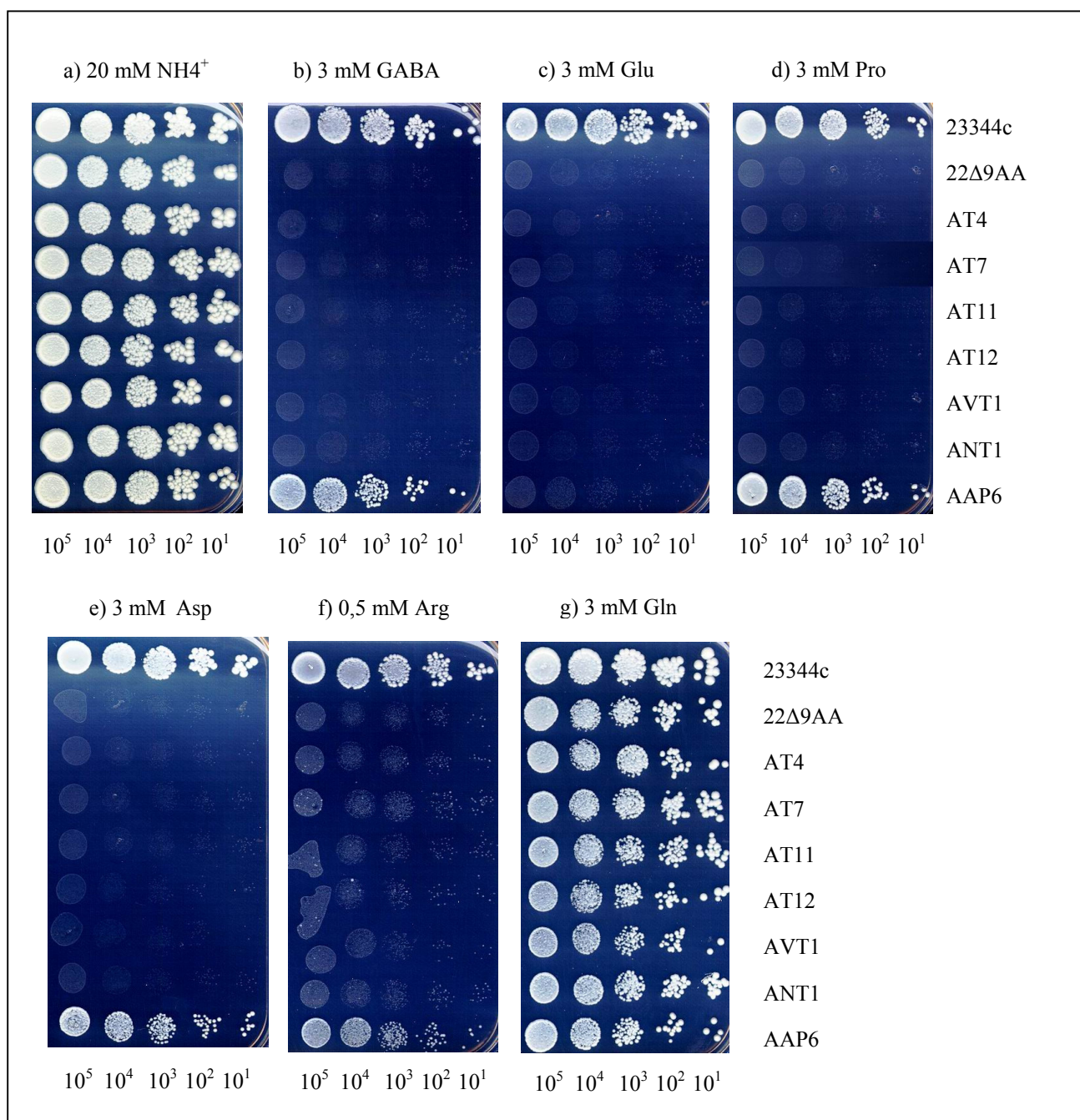
Figure 9 – Complementation of the 22Δ9AA strain. 3 days growth on BA medium supplemented with a) 20 mM NH_4^+, b) 3 mM gamma amino butyric acid, c) 3 mM glutamic acid, d) 3 mM proline, e) 3 mM aspartic acid, f) 0,5 mM arginine, g) 3 mM glutamine. Approximately 10^5, 10^4, 10^3, 10^2 or 10^1 cells were applied on each spot (from left to right). From top to bottom in each section, growth of wild type 23344c strain, growth of 22Δ9AA strain transformed with the empty vector (pDR195) and with pDR vector containing AtAT4, AtAT7, AtAT11, AtAT12, ScAVT1, AtANT1 or AtAAP6 gene.

3.2.3. Complementation assay of the JT16 strain with the ATs

The JT16 strain is impaired in histidine transport, as the histidine permease gene *hip1* is deleted, as well as the histidine and arginine permease gene *can1*. It is also auxotrophic for histidine, as the histidinol dehydrogenase gene *his4* is deleted (Tanaka and Fink, 1985). This strain is unable to grow unless a sufficient amount of histidine (30 mM in SC medium) is supplemented in the medium. It has been used as a complementation system to isolate or characterize new amino acid transporters (Chen & Bush, 1997, Chen *et al.*, 2001, Fischer *et al.*, 1995, Frommer *et al.*, 1995, Hsu *et al.*, 1993), using lower concentrations of histidine: 6 mM in SC medium; AtCAT1 is able to grow also on 0.6 mM His (Frommer *et al.*, 1995). The ability of AT4, AT7, AT11 and AT12 to complement the inability to grow on medium with a limited supplement of histidine was analyzed. AtANT1 and AtCAT1 were chosen as positive controls. None of the AT genes was able to complement the histidine transport deficiency in JT16 and under our conditions AtANT1 was also not able to complement the mutations, while AtCAT1 was able to complement as expected (Fig. 10).
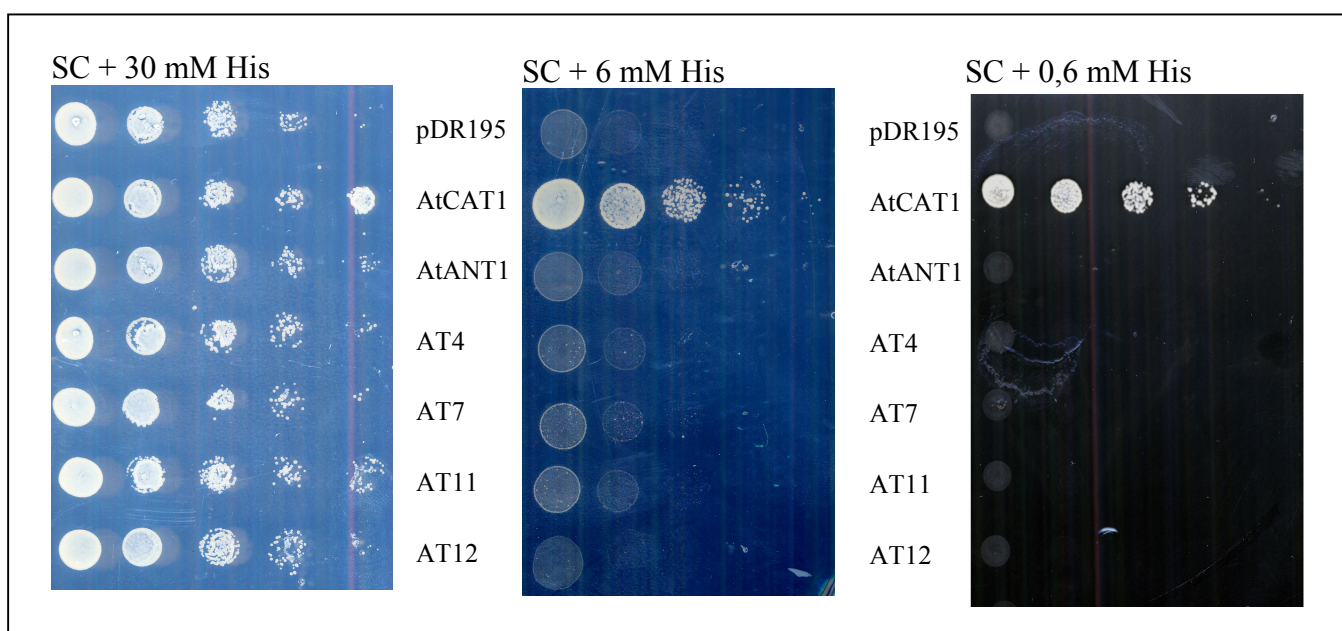
Figure 10 – Complementation of the JT16 strain. 3 days growth on SC medium supplemented with 30 mM His (left), 6 mM His (middle) or 0,6 mM His (right). Approximately 10^5, 10^4, 10^3, 10^2 or 10^1 cells were applied on each spot (from left to right). From top to bottom in each section, growth of JT16 strain transformed with the empty vector (pDR195) and with pDR vector containing AtCAT1, AtANT1, AtAT4, AtAT7, AtAT11 or AtAT12 genes.

3.2.4. Uptake of radioactive histidine in JT16 yeast strain mediated by AT4, AT7, AT11

To confirm the negative results of the complementation test with the JT16 yeast mutant strain, considering that the known transporter AtANT1 did not complement under test conditions, it was decided to measure the direct uptake of histidine in yeast cells. The uptake in yeast expressing AT1, AT7 and AT11 was compared with the uptake in yeast expressing AtCAT1. In both cases the plasmid containing the coding sequence for AT proteins were not able to mediate uptake of histidine, as compared to the control (empty vector), while the positive control (AtCAT1) was able to uptake histidine in all cases (Fig. 11).

Figure 11 – Uptake of radiolabelled histidine in JT16 yeast cells transformed with pDR195 or pDR expressing AT4, AT7, AT11 and AtCAT1.

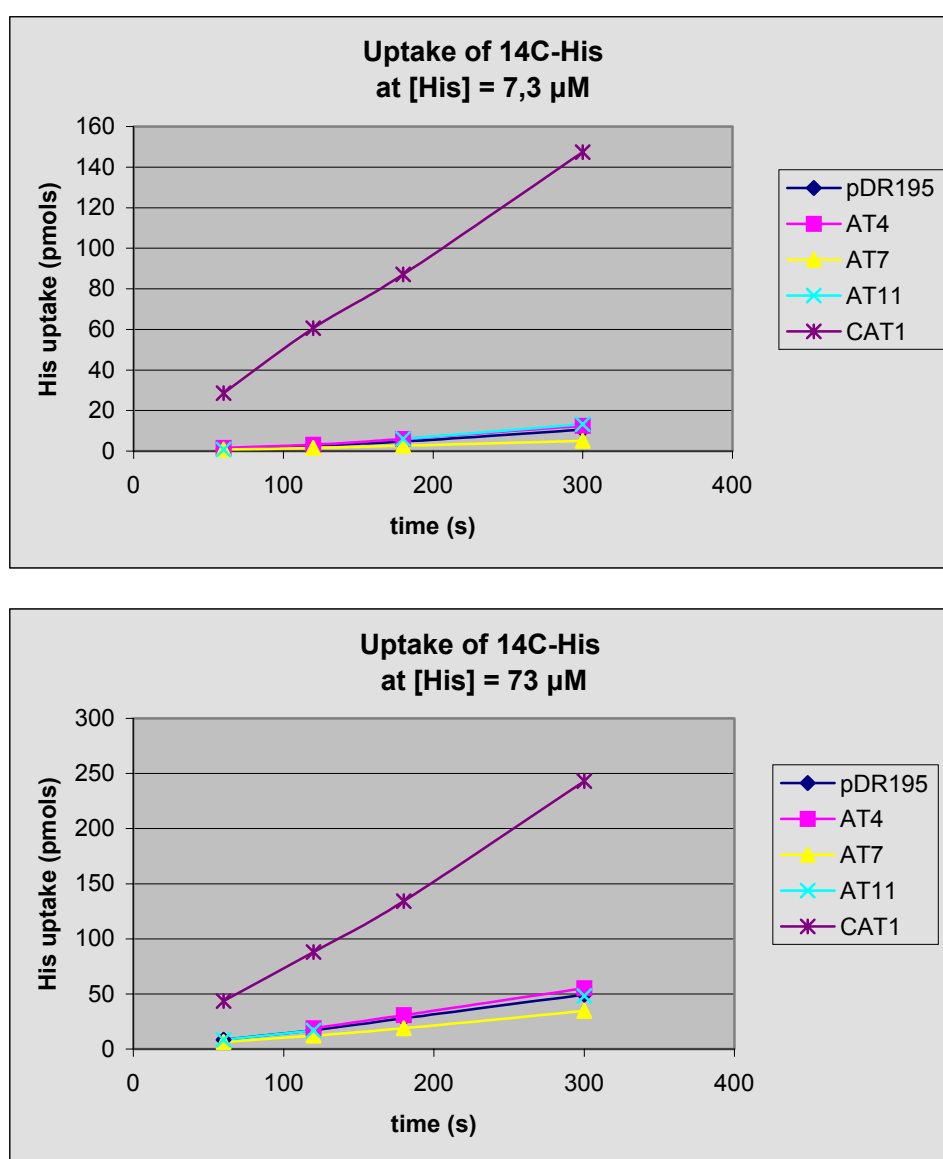3.2.5. Development and use of the G119 yeast strain

To find and characterize novel amino acid transporters localized on the tonoplast, a new yeast strain, G119, was developed. The concept of this strain is schematized in Figure 15.

This strain is based on the idea of expressing the asparaginase activity in the vacuole. If the tonoplast localized asparagine importer is knocked out, the strain could be used to screen for vacuolar importers of asparagine. Therefore, the known gene for the vacuolar transporter *avt1*, which transports asparagine (Russnak et al., 2001), was knocked out.

This *asp1* gene was then deleted in this strain that is therefore unable to use asparagine as the sole nitrogen source (Fig. 12)
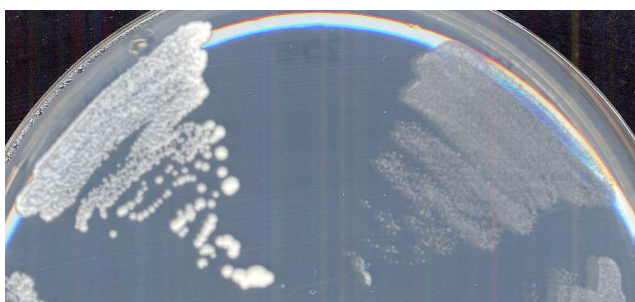
Figure 12 – Comparison of the growth of the strain Δavt1 (left) and the strain G119 before insertion of the YCPlac-V/asp plasmid (right) on BA medium supplemented with 5 mM asparagine. The deletion of asp1 renders the strain unable to grow on asparagine as the sole nitrogen source.

G119 strain carries a plasmid (YCPlac-V/Asp) containing the promoter and the leader sequence of the carboxypeptidase gene (*cpy*) fused in frame to the coding sequence of the *asp3* gene, that codes for an asparaginase which works outside the yeast cell. The *asp3* gene is not present in the 23344c wild type strain. The *cpy* targeting sequence should enable the vacuolar localization, the *asp3* gene should provide a protein which is able to work under the acidic conditions that are present in the vacuole.

There is also a deletion in the *pep4* gene in this strain. Mutants of this gene lacks most of the hydrolase activity in the vacuole (Jones *et al.*, 1982, Klionsky & Emr, 1989); this should help to stabilize the asparaginase activity in the vacuolar environment.

A possible mislocalization in the cytoplasm of the activity encoded by *asp3* was excluded as in this case the strain would be able to grow on asparagine as the sole nitrogen source (Fig. 13).
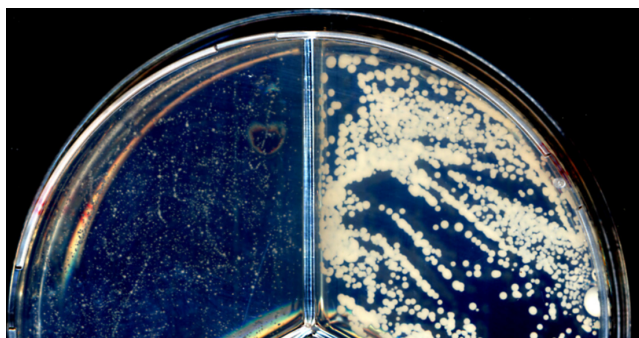
Figure 13 – Comparison of the growth of the strain G119 (left) and the growth of the strain 23344c (right) on BA medium supplemented with 5 mM asparagine. The plasmid YCPlac-V/Asp does not restore the ability to grow on asparagine as sole nitrogen source – i.e. the asparaginase activity is not mislocalized in the cytoplasm.

Unfortunately, the strain was unable to grow on asparagine as the sole nitrogen source even when transformed with pDR-AVT1, a plasmid that carries the *avt1* gene (Fig. 14). Therefore, it can be supposed that either the vacuolar asparaginase was inactive, or that ScAVT1 is actually unable to transport asparagine in the vacuole. A recent publication suggested that the AVT proteins might not actually be localized to the tonoplast or only under undetermined specific conditions which could explain this result (Prusty *et al.*, 2004).

The strain was tested with AT4, AT7, AT11 and AT12, determining if these genes were able to support the growth of the strain coding for asparagine transporters in the vacuole. None of these genes complemented the mutations of G119, at least not under the conditions of the test (Fig. 14).
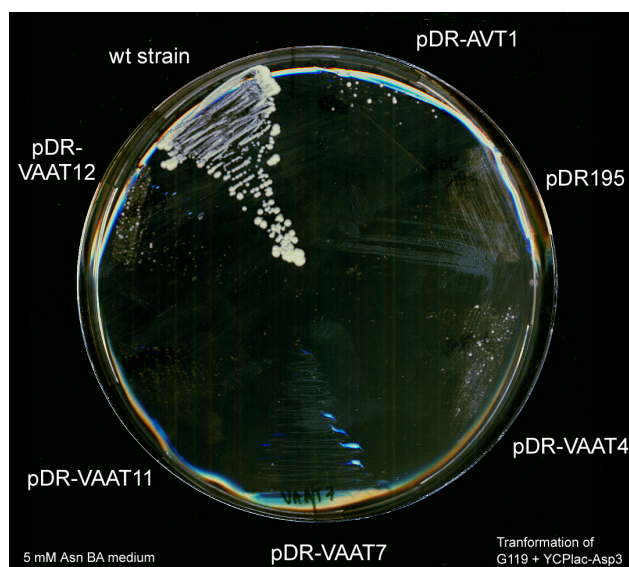
Figure 14 – 3 days growth on BA medium with 5 mM Asn as the nitrogen source of the wild type 23344c strain (wt) and of the G119 strain transformed with an empty vector (pDR195) or expressing ScAVT1 (pDR-AVT1), AT4, AT7, AT11 or AT12. None of the tested genes was able to support the growth of the strain under the conditions tested.

A way to improve the system could be to delete a further gene, *asp5*, that codes for the Aspartate Aminotransferase 2. The resulting strain would be aspartate auxotrophe and asparagine could be given as sole source of aspartate, instead of source of nitrogen.
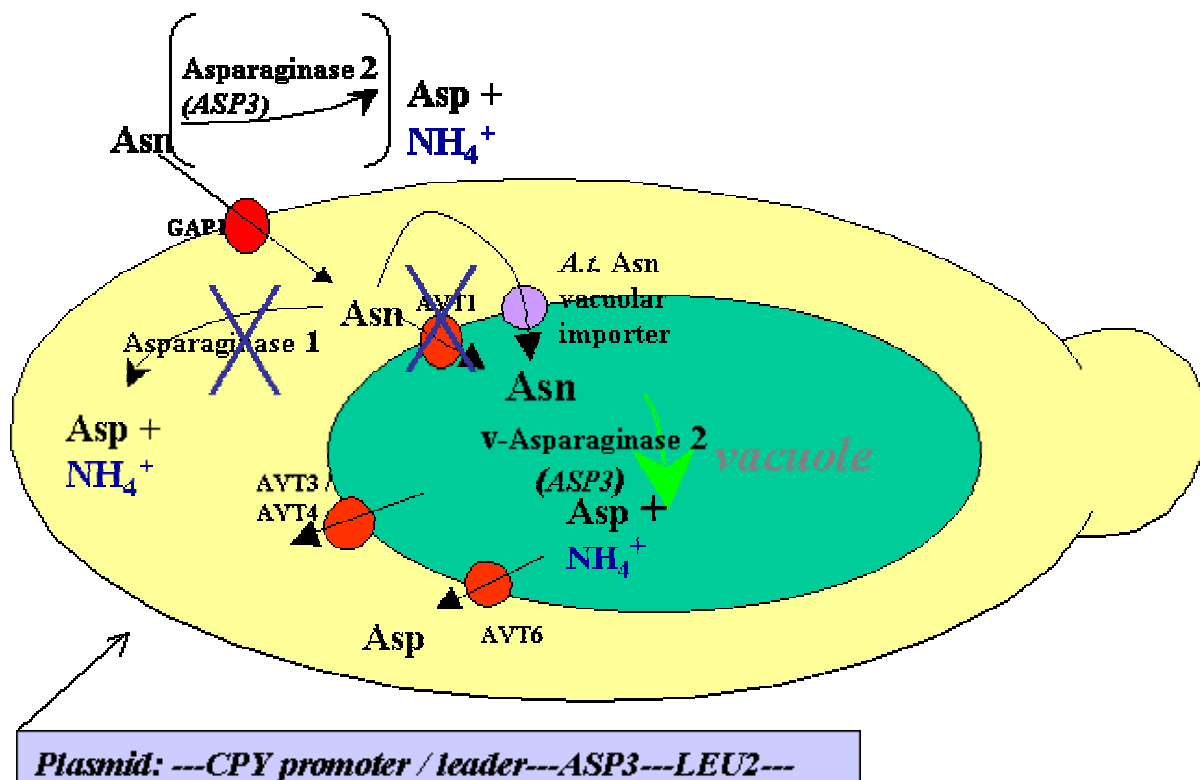
Figure 15 – Schematic model of the G119 strain and its use to isolate and characterize new vacuolar amino acid transporters. The *asp1* gene is deleted and no asparaginase activity is present in the cytosol. The strain is, therefore, unable to grow on asparagine as the sole nitrogen source. The asparaginase activity was transferred to the vacuole via a plasmid that contains the *asp3* gene under the control of the CPY promoter and leader sequence. The gene for vacuolar importer of asparagine *avt1*, was also deleted.

3.2.5. Effect of toxic amino acid analogs

The sensitivity to different toxic analogs of amino acids in yeast expressing AT4, AT7, AT11 and AT12 and different controls was tested. An increase of sensitivity would suggest a role of the respective putative transporter as importer of amino acids, while a decrease would suggest a role as exporter or a sequestering of the substance in the vacuole via a tonoplast localized importer.

3.2.5.1. Effect of 6-diazo-5-oxo-norleucine

6-diazo-5-oxo-norleucine (DON) is a toxic amino acid, whose structure is similar to Gln. It is recognized by the GlutamineOxoGlutarateAminoTransferase as substrate (Lebedeva *et al.*, 1986). As expected, the wild type 23344c yeast was clearly inhibited in growth when cultured on BA medium with a concentration of DON of 150-200 nM (Fig. 16). Under the same conditions, the 22∆8AA strain shows normal growth also at a concentration of 250 nM DON.

We expected that if one of the tested constructs was able to detoxify the compound, this would be due to export or sequestering in the vacuole of DON mediated by a plasma membrane exporter or a vacuolar importer of amino acids. However, yeasts of the 23344c strain expressing AT4, AT7, AT11, AT12 were not influenced in their sensitivity to the toxic compound (data shown for AT7, Fig. 16).
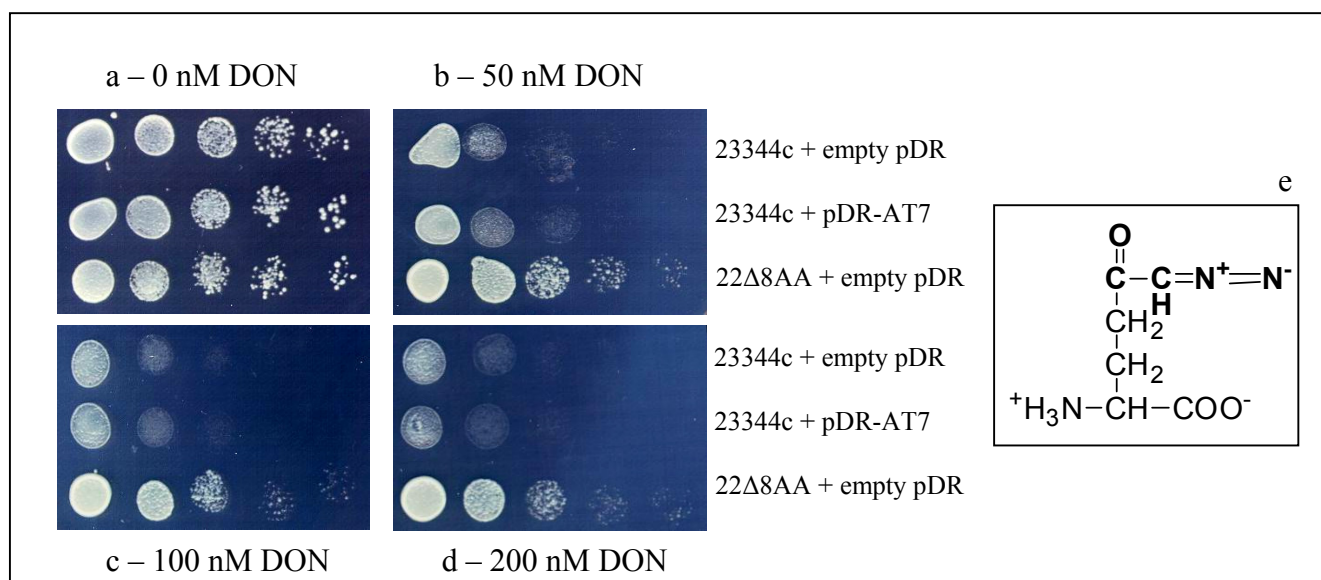
Figure 16 – Growth after 3 days of incubation at 27°C. a,b,c,d At each spot from left to right about 10^5, 10^4, 10^3, 10^2, 10^1 cells were applied. In the upper row: 23344c strain transformed with an empty pDR vector; in the middle row: 23344c strain transformed with a pDR vector containing the coding sequence for AT7 (pDR-AT7); in the lower row: 22Δ8AA strain, transformed with an empty pDR vector. In e the structural formula of 6-diazo-5-oxo-norleucine (DON).

3.2.5.2. Effect of 5-methyl-tryptophane

5-methyl-tryptophane (Fig. 17 a) is a toxic analog of tryptophane that causes inhibition in an enzyme of the metabolic pathway of tryptophane biosynthesis, the anthranylate synthase. It causes tryptophane starvation as it acts as a false feedback inhibitor (Schurch *et al.*, 1974, Zhang *et al.*, 2001).

While testing for the toxic concentration of 5-mT, another interesting property was found and used instead of the toxicity to screen the AT genes for the transport of amino acids. Under concentrations below the toxicity level (2 mM in SD medium), the color of the yeast colonies turned yellow which became red after exposure to light for several days. A clear difference was noticed between the color of the colonies of yeast transformed with an empty vector and the colonies of yeast transformed with an efficient transporter of tryptophane such as AtAAP6, that caused a stronger staining of colonies (Fig. 17 b). This is probably due to accumulation of a degradation product of 5-mT and is, therefore, an indication of the transport of this substance.

A color assay was therefore developed. The assumption was that if a gene is able to alter the color of the colonies towards yellow/red more than the negative control (empty vector), the gene might encode a carrier able to mediate the accumulation of 5-mT and probably therefore also transport tryptophane. Yeast transformed with the ATs were tested for color changes more intense than in the yeast transformed with the empty vector. No difference in the color changes were however observed in the yeast expressing the ATs, while the color shifted towards yellow/red in the yeast expressing the positive control AtAAP6. This might be an indication that tryptophane is not transported by these putative transporters.
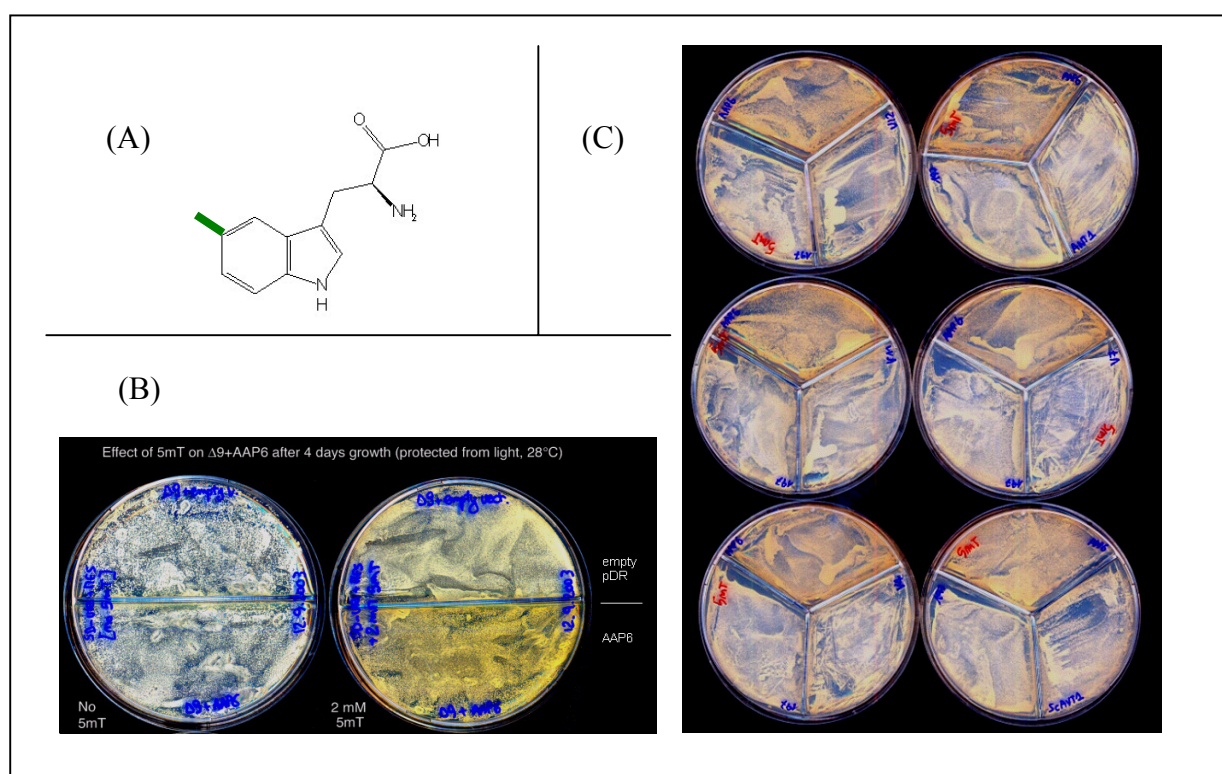
Figure 17 – Effect of 5-mT on the yeast colonies with concentrations below the toxicity level. The yeast was grown on SD-ura medium additioned with 2 mM of 5mT. At this concentration in the SD medium the yeast is still able to grow but turns yellow when protected from the light (B) or red when exposed to the light (C). (A) the formula of 5-methyl-tryptophane. (B) Comparison between growth on SD-ura medium (on the left) without 5-mT and the same with 2 mM 5-mT (on the right), of 22Δ9AA strain transformed with an empty vector (top) or with pDR-AAP6 (bottom). (C) Test for ATs: on each plate in the top compartment 22Δ9AA transformed with pDR-AAP6, in the bottom left, 22Δ9AA transformed with pDR195, in the bottom right, 22Δ9AA transformed with the test vector. The test vector was pDR containing AT12 (left), AtANT1 (right) in the first row, AT11 (left), AT7 (right) in the second row, AT4 (left), ScAVT1 (right) in the third row.

3.3. Analysis of AT4, AT5, AT9 and AT12 promoter activities.

The *uidA* gene of *Escherichia coli*, coding for β-glucuronidase (GUS) was used for studies on the functionality of the promoters and for localization of the expression driven by a promoter in specific organ and tissues. The GUS activity in a specific location monitors the expression of the gene in that location. The activity of the promoter of the AT4, AT5, AT9 and AT12 genes was analyzed by histochemical localization of GUS activity in transgenic plants transformed with a construct consisting of *uidA*, under the transcriptional control of each promoter of these genes.

3.3.1. AT4 promoter activity

The region between the end of the known 3'-UTR of the previous gene on the chromosome (At5g02160) and the ATG of the AT4 gene was chosen as maximal promoter region of the gene AT4. The region is 604 bp long. The 5'-UTR region of the AT4 gene is included in the construct.

Promoter activity was observed in hypocotyls of seedlings 2 days after the germination, but not in other parts of the seedling like roots or cotyledons (Fig.18 a). The activity in the hypocotyl decreases with the age of the plant. In older plants, however, promoter activity was observed in the petiole of all leaves, except for the cotyledons (Fig.18 b). This activity also decreases in intensity with the age of the plant. There was no observable activity in the reproductive organs.

a)   b)
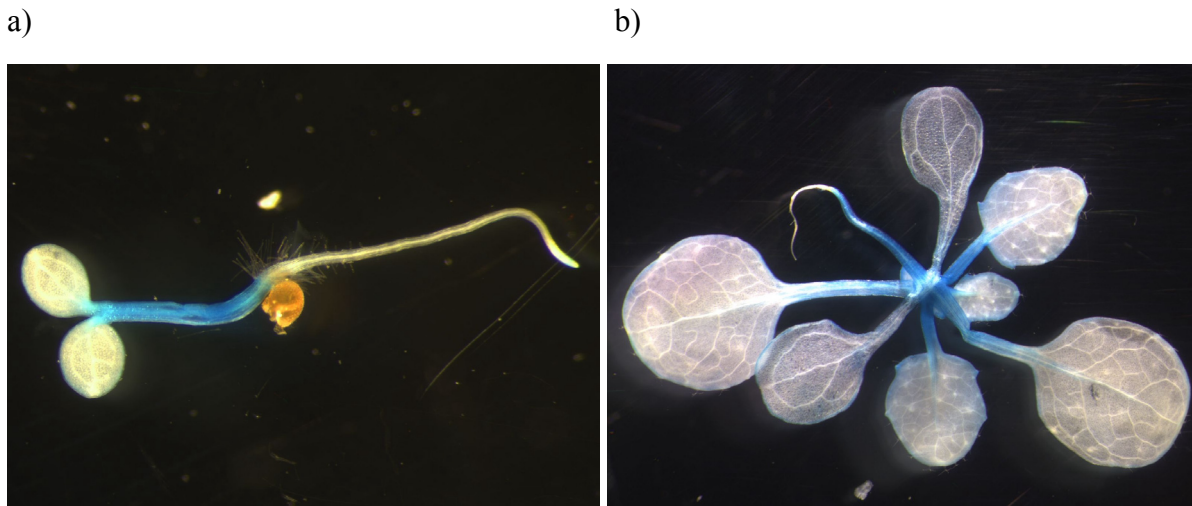
Figure 18 – a) on the left, promoter activity of AT4 in a seedling 3 days after germination on MS agar plate. The activity is localized in the hypocotyl region. b) on the right, promoter activity of AT4 in a seedling about 4 weeks after the germination in soil in the greenhouse. The promoter activity is still localized in the hypocotyl region, but there is also activity in the petiole of true leaves.

3.3.2. AT5 promoter activity

The region between the ATG of the previous gene (that codes on the opposite strand) on the chromosome (At2g41200) and the ATG of the AT5 gene was chosen as the maximal promoter region of the AT5 gene. The region is 1511 bp long. The 5'-UTR region of the AT4 gene is also included in the construct. No promoter activity was observed under standard growth conditions, i.e. seedlings grown in MS agar plates or in soil in the greenhouse (data not shown).

3.3.3. AT9 promoter activity

An upstream region of about 2 kilobases (1973 bp) between the previous gene on the chromosome (At2g39140) and the AT9 gene, up to the ATG of the AT9 gene, was chosen as the maximal promoter region of the AT9 gene. The 5'-UTR region of the AT9 gene is therefore included in the construct. Promoter activity was observed in young seedlings in the hypocotyls, in the cotyledons and in the roots (Fig. 19 a) and in older plants in leaves, especially in the vascular system (Fig. 19 e) and in the lateral roots (Fig. 19 b). Activity was

also present in the reproductive organs such as the pollen grains, the anthers and the stigma (Fig. 19 d), as well as at the tip and the base of the siliques (Fig. 19 c).

a

b

c

d

e
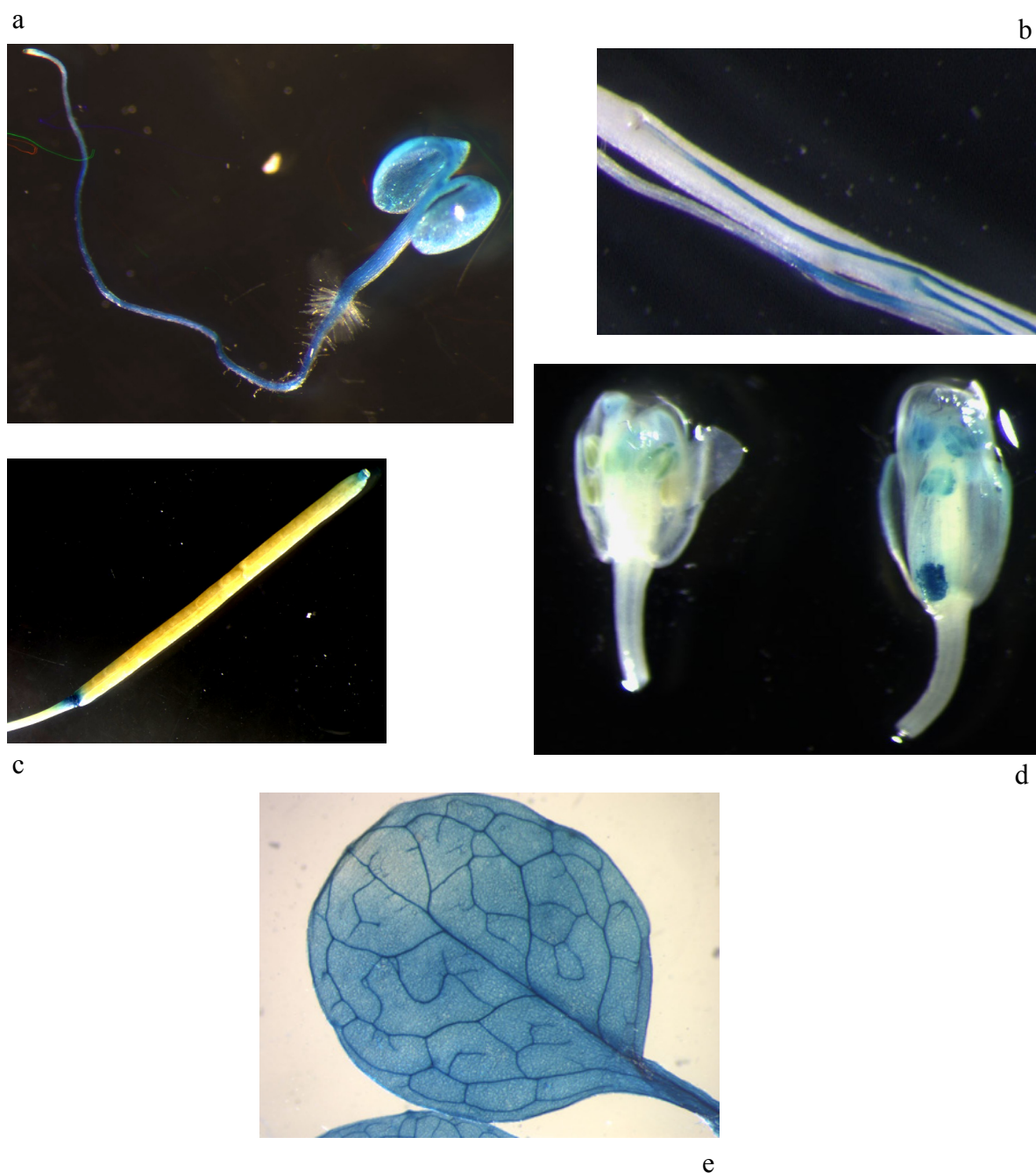
Figure 19 – Histochemical staining of GUS activity under the control of the AT9 promoter. (a) a 5 day old seedling is stained in the root (except root hairs), hypocotil and cotyledons. (b) root of a 6 week old plant grown in soil. There is staining of the lateral roots. (c) silique (staining at the bottom and at the top). (d) flowers. The staining is in petals, in the sigma and in the pollen grains in the anthers. (e) cotyledon of a 3 weeks old plant. The staining is more intense in the vascular system.

3.3.4. AT12 promoter activity

The region between the stop codon of the previous gene on the chromosome (At5g65980) and the ATG of the AT12 gene was chosen as the putative maximal promoter of the AT12 gene. The region spans 722 bp. The 5'-UTR region of the AT12 gene is also included in the construct. Promoter activity was observed in the hypocotyl of young seedlings and in cotyledons but not in the roots (Fig. 20 a,b,c). Staining was observed in older plants in leaves (Fig. 20 c), more intense in cotyledons and with decreasing intensity in the following young leaves. Activity is also present in the reproductive organs such as pollen grains, in the anthers and the stigma (Fig. 20 d) as well as at the base and the tip of the siliques (Fig. 20 e).
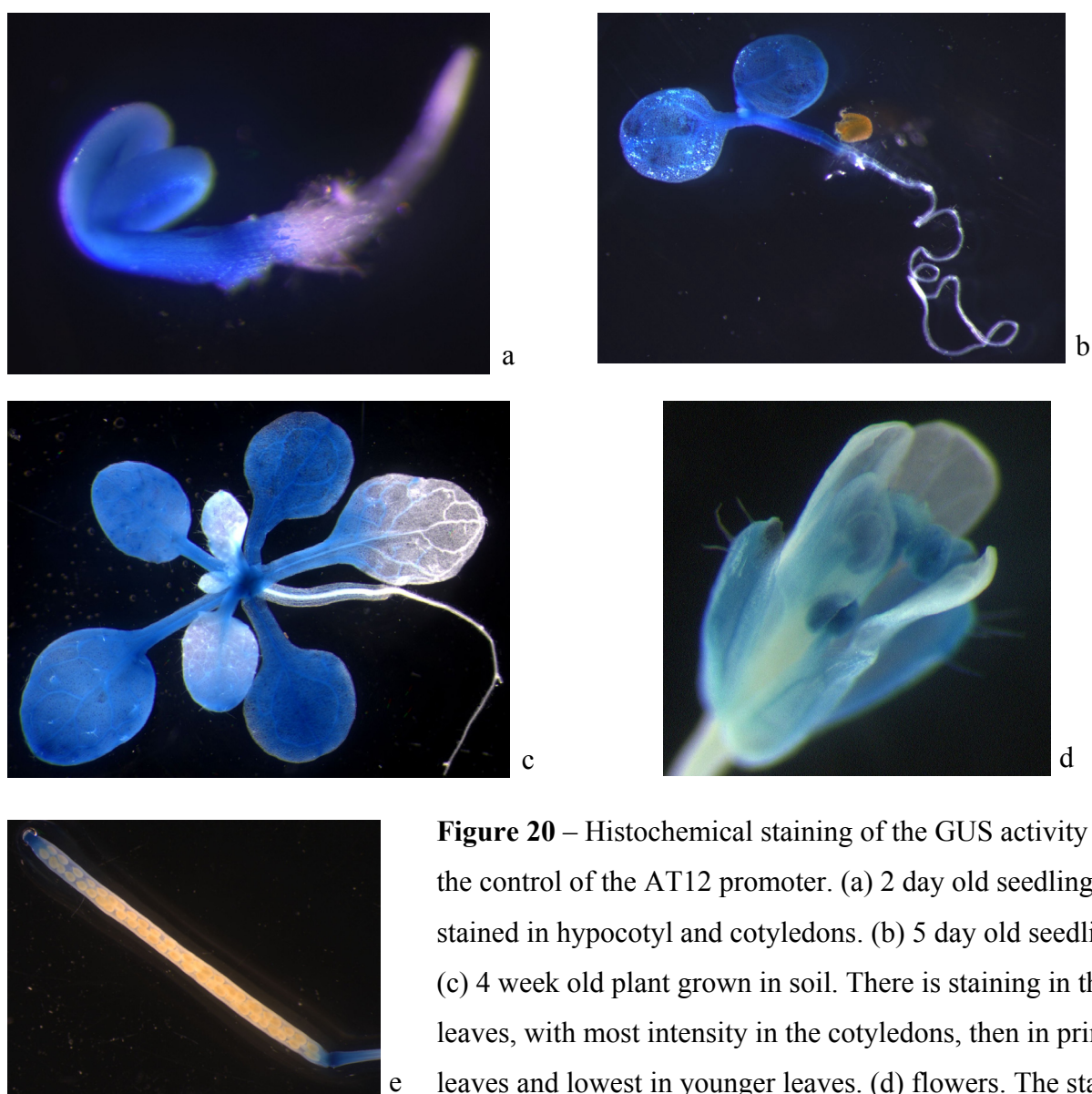
Figure 20 – Histochemical staining of the GUS activity under the control of the AT12 promoter. (a) 2 day old seedling is stained in hypocotyl and cotyledons. (b) 5 day old seedling (c) 4 week old plant grown in soil. There is staining in the leaves, with most intensity in the cotyledons, then in primary leaves and lowest in younger leaves. (d) flowers. The staining can be seen in petals, in the sigma and pollen grains. (e) silique. There is staining at the bottom and at the top of the silique.

3.4. Subcellular localization by protein fusion with GFP

The intracellular localization of a transporter is important for understanding the function of the transporter in the cell and locate the compartment where the transport occurs. To identify the membrane in which the ATs are active, fusions of green fluorescent protein (GFP) with the ATs were expressed in protoplasts of *Arabidopsis thaliana*.

Since the orientation of the N- and C-termini may be crucial for the detection of the fused GFP, the transmembrane prediction by TMHMM for each protein was used to select which termini would be more suited to construct the fusions.

3.4.1. GFP-AT1 and AT12-GFP fusions are expressed in endomembranes of *Arabidopsis* protoplasts

AT1 has a predicted N-terminus in the cytoplasm. It was therefore decided to fuse the protein with an N-terminal GFP. The AT1 CDS was fused in frame with the 3' terminus of the GFP CDS without the stop codon. The GFP-AT1 fusion was transiently expressed in protoplasts under the control of the 35S promoter.

AT12 has a predicted C-terminus in the cytoplasm and a predicted extracellular N-terminus. It was therefore decided to fuse the protein with a GFP at the C-terminus. The AT12 CDS without the stop codon was fused in frame with the 5' terminus of the GFP CDS. The AT12-GFP fusion was transiently expressed in protoplasts under the control of the 35S promoter.

Transformed protoplasts with the AT1 and AT12 fusions were analyzed by UV confocal microscopy at 24, 48 and 72 hours after transformation. Fluorescence was seen in the interior of the cells, outside of the vacuole (Fig. 21). The observed pattern is clearly differentiable from the one of soluble proteins such as GFP, which was used as control for soluble proteins (Fig. 21). The probable localization of the fusions is therefore in endomembranes, such as the endoplasmic reticulum.

3.4.2. GFP-AT11 fusion is not expressed in *Arabidopsis* protoplasts.

Both the termini of AT11 are predicted to be localized in the cytoplasm. It was decided for convenience of cloning to fuse the protein with an N terminal GFP. The AT11 CDS was fused with the 3' terminus of the GFP CDS without the stop codon. The GFP-AT11 fusion was transiently expressed in protoplasts under the control of the 35S promoter. Transformed

protoplasts were analyzed by UV confocal microscopy at 24, 48 and 72 hours after transformation. They did not show any detectable expression.
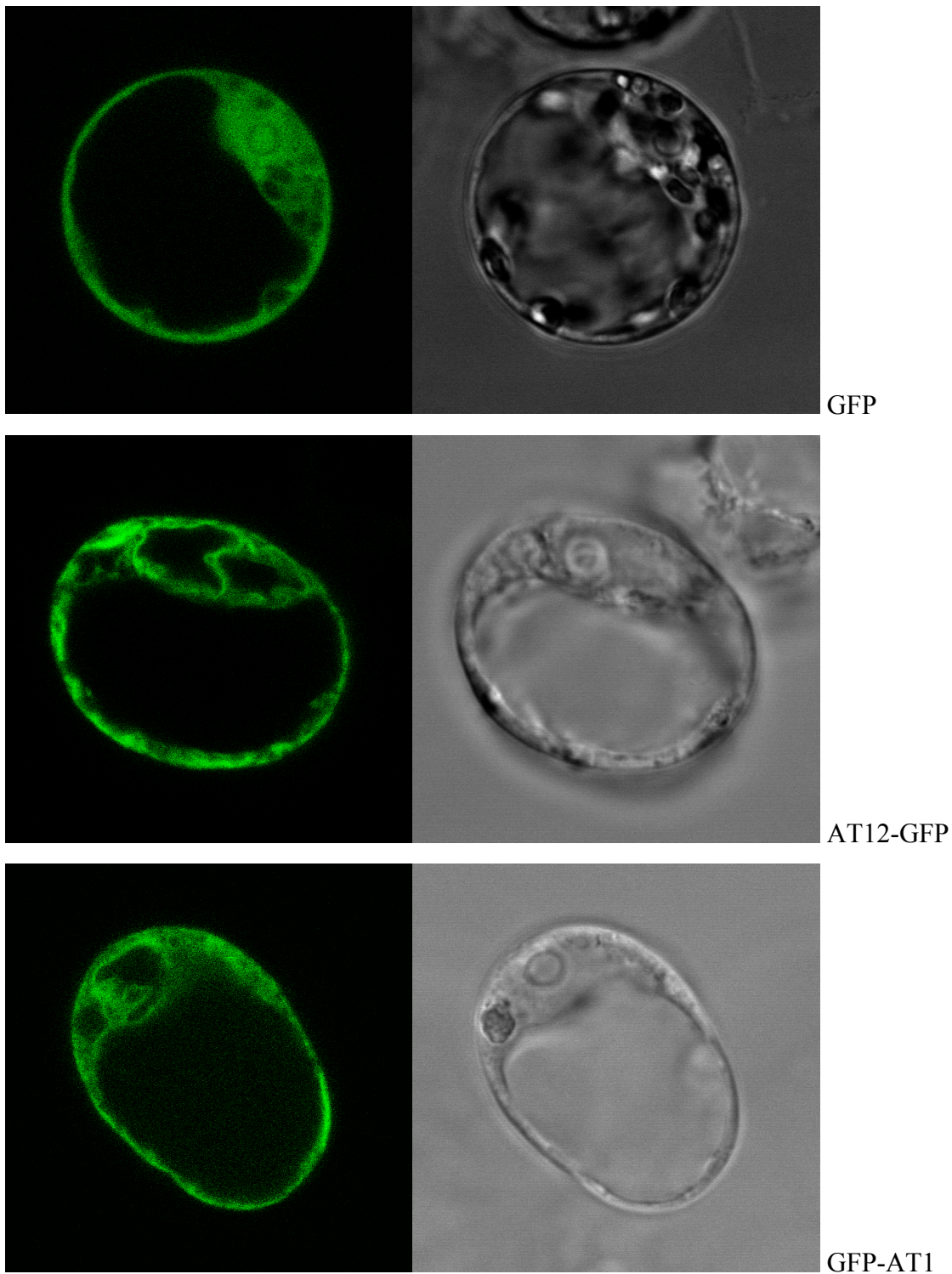

GFP

AT12-GFP

GFP-AT1

Figure 21 – Expression in *Arabidopsis thaliana* protoplasts of the AT12-GFP fusion (2nd row) and the GFP-AT1 fusion (3rd row)**,** in comparison with the cytoplasmic expression of GFP (1st row). On the left side the fluorescence image, on the right transmission image of the same protoplast.

3.5. T-DNA insertion lines

T-DNA insertion lines are a powerful tool to characterize the function of a gene by analyzing the effect of the disruption of a particular gene on the phenotype of a plant. To analyze the function of the AT genes, T-DNA lines with insertion in the AT genes were ordered from GABI (Rosso *et al.*, 2003) and from SALK Institute (Alonso *et al.*, 2003) collections (Table 8). Recently other T-DNA collections have been made available: Table 9 reports a list of the currently available T-DNA lines per each AT gene.

Ten single plants were isolated from each line and the phenotype compared with the wild type plants planted at the same time. To assess if some of these plants were homozygous for the T-DNA insertion we extracted the genomic DNA from the plants and analyzed them by PCR. Such homozygous lines, where the T-DNA is inserted in both copies of the gene were found in all cases. A full characterization of the T-DNA lines would have implied backcrossing and confirmation of the sequence of insertion of the T-DNA, that has not been completed.

No variation in viability, shape of the plant, number and size of leaves, flowers, secondary stems or in germination and flowering time was observed when plants were grown under standard conditions (data not shown). The AT7 insertion line, however, made an exception.

Gene	Accession	Position	Homozygous
AT1	GABI 368G05	Exon	3
AT4	SALK 090177	Intron	7
AT5	SALK 087204	Exon	3
AT7	GABI 110D04	Exon	3
AT9	SALK 068456	Intron	3
AT12	SALK 010447	Exon	5

Table 8 – T-DNA lines analyzed for each AT gene. Entry code for GABI and SALK collections are given in the second column. SALK indicate a line from the Salk Institute T-DNA lines collection (Alonso *et al.*, 2003), GABI from the GABI Project T-DNA lines collection (Rosso *et al.*, 2003). The third row indicates whether the position of the insertion is in an exon or an intron of the gene. In the fourth row the number of homozygous plants identified among 10 analyzed plants is indicated.

3.5.1. Homozygous plants for T-DNA insertion in the AT7 gene are sterile

While the heterozygous plants for the AT7 T-DNA insertion showed as expected no particular phenotype, homozygous plants were sterile. Further examinations are needed to determine if this sterility is due to pollen or maternal sterility.

Gene	MIPS name	Entry codes of available T-DNA lines
AT1	At5g02180	SM_3_40199 (E), SM_3_41002 (E), GABI_518A05 (E), SM_3_40347 (E), SAIL_210_H05 (I), GABI_560F04 (E)
AT2	At3g09340	SM_3_29107 (E), SM_3_29093 (E), FLAG_595C07 (E), SM_3_35227 (E), SM_3_35631 (E), SM_3_35230 (E), SM_3_35233 (E), SM_3_15537 (E), FLAG_104B11 (I), SM_3_20778 (I), SM_3_38598 (I), SM_3_38604 (I), SM_3_15548 (E), CSHL_GT2104 (E), SM_3_465 (I), CSHL_GT2007 (E), RATM16-2145-1_G (E), SM_3_28648 (I), SM_3_20159 (E), SM_3_20161 (E), SM_3_40107 (E), GT_5_112574 (E), GT_5_112471 (E), RATM11-2240-1_H (E)
AT3	At3g09330	RATM13-2061-1_G (I), SM_3_967 (I), RATM12-0623-1_G (E)
AT4	At5g02170	GT_5_54073 (E), GT_5_53499 (E), GABI_527B09 (E), WiscDsLox244E02 (I), SALK_090177.45.90.x (I), SALK_090178.29.10.x (I), RATM16-0796-1_H (E)
AT5	At2g41190	GABI_341C12 (E), GABI_147C12 (I), SALK_010269.46.95.x (I), SALK_087204.54.00.x (E), SALK_058332.48.80.x (I), SALK_058332.52.30.x (I)
AT6	At3g28960	SAIL_112_A12 (E)
AT7	At5g15240	SALK_020258.48.40.x (I), SALK_020273.54.75.x (I), SM_3_41209 (I), SAIL_174_C10 (I), SALK_113877.37.30.x (E)
AT8	At3g54830	SAIL_1291_E10 (E), WiscDsLox335B10 (E)
AT9	At2g39130	GABI_715A07 (E), SALK_068456.36.40.x (I), SAIL_106b_C06 (E), SALK_134270.26.40.x (I), SALK_027465.26.40.x (I), SAIL_594_F02 (E), RATM16-1544-1_H (E)
AT10	At5g16740	GABI_483C02 (E), SALK_016987.12.35.x (E), SALK_016891.15.20.x (E), RATM52-0851-1_H (I), GABI_856B06 (E), WiscDsLox441G6 (E), WiscDsLox420A01 (E), RATM52-0851-1_G (I), SALK_067040.51.70.x (E), SALK_067040.55.75.x (E), FLAG_529H06 (E), FLAG_393G06 (I), SAIL_1240_E08 (I), SM_3_30773 (E)
AT11	At4g38250	RATM16-1969-1_G (E), CSHL_ET6266 (E), SAIL_749_D04 (E), RATM16-1969-1_H (E)
AT12	At5g65990	SALK_010447.43.10.x (E), GT_5_111320 (E), SALK_028557.56.00.x (E)
AT13	At2g42005	GABI_099E04 (E), SAIL_766_C07 (E), CSHL_GT7496 (E), SM_3_24312 (E), SALK_063680.56.00.x (E), CSHL_GT7496 (E), SALK_025674.43.20.x (E), SALK_063672.55.25.x (E), SAIL_615_E01 (E)

Table 9 – List of the T-DNA lines currently available for each AT gene (from the Signal SALK database: http://signal.salk.edu/). In the first column the gene name is indicated, in the second the MIPS entry name, in the third a list of T-DNA lines available. Location of the insertion: (E): exon (I): intron. The beginning of the name indicates to which collection the line belongs: SM or GT = SM Line (Tissier *et al.*, 1999), GABI = GABI-Kat (Rosso *et al.*, 2003), SAIL = SAIL FST (Sessions *et al.*, 2002), FLAG = FLAG FST (http://flagdb-genoplante-info.infobiogen.fr/projects/fst/), CSHL = Cold Spring Harbour Laboratory FST (http://genetrap.cshl.org/), RATM = RIKEN FST (Kuromori *et al.*, 2004), Wisc = Wisc FST (http://www.hort.wisc.edu/krysan/DS-Lox/), SALK = SALK T-DNA (Alonso *et al.*, 2003).

4   Discussion

4.1. Analysis of sequence similarities and predicted protein structures reveals new putative amino acid transporters

The genes for new putative amino acid permeases, named AT1 to AT13 (Amino acid Transporter 1 to 13), have been identified in the *Arabidopsis thaliana* genome. *Arabidopsis thaliana* is the first plant for which the genome has been completely deciphered (Arabidopsis Genome Initiative, 2000) and this has allowed the use of a genome wide approach for the *in silico* identification of new amino acid transporters.

These proteins are classified by homology as putative transporters and it is possible to suggest their substrate specificity by comparison with known related transporters (Tables 2,3,4). Membrane proteins are classified as extrinsic or intrinsic: the first group is formed by proteins which are only associated to the surface of the lipid bilayer, while the second group contains proteins, also called transmembrane proteins, that have hydrophobic regions spanning across the lipid bilayer. Among the transmembrane proteins are receptors involved in signaling cascades and pathogen defense reactions, enzymes such as the apparatus for cell wall biosynthesis, and transporters responsible for the import and export of solutes and ions and the establishment of electrochemical gradients across membranes. Thereby they connect the different metabolic pathways of the cellular compartments and organelles. Transmembrane proteins with an elevated number of putative transmembrane domains, that have been functionally characterized, are either permeases or membrane receptors. A complete set of the transmembrane proteins with one or more transmembrane domains according to several TM prediction programs has been summarized in the Aramemnon database (Schwacke *et al.*, 2003). Using this tool, a BLAST in the *Arabidopsis* proteome was performed, limited to the proteins classified as transmembrane, to find which proteins are related to known transporters.

The AT proteins belong to the ATF1 superfamily. A phylogenetic tree of all related proteins belonging to the ATF1 superfamily was constructed (Fig. 7). The amino acid transporters that have been isolated and characterized in *Arabidopsis thaliana* belong to two superfamilies. AtCAT1 (Frommer *et al.*, 1995) belongs to the APC (amino acid - polyamine – choline) superfamily. All the other functionally characterized transporters in *Arabidopsis* belong to the ATF1/AAAP (amino acid – auxin permease) superfamily and consist in the AtAAPs (Fischer *et al.*, 2002, Okumoto *et al.*, 2002), AtProT1 and AtProT2 (Breitkreuz *et al.*, 1999, Rentsch *et al.*, 1996), AtLHT1 (Chen & Bush, 1997) and AtANT1 (Chen *et al.*, 2001). Many of the

known amino acid transporters have been identified by the suppression of yeast mutants (Frommer & Ninnemann, 1995). Several proteins related to the known amino acid transporters have been identified. Besides the proteins directly related to the known Arabidopsis transporters other related proteins are homologs of the yeast members of the ATF1 family (Table 2, 3, 4) and are as well uncharacterized. This study concentrated on two subfamilies of these proteins. The first subfamily consists of the proteins AtAT1 to AtAT10 and contains proteins whose closest relative with an assigned function is ScAVT1. The second subfamily consists of AtAT11, 12 and 13, whose closest relatives are AtANT1, ScAVT3 and ScAVT4.

All the AT proteins have a high number of transmembrane domains (Table 5). The AT11/12/13 family has a common hydrophobicity profile (Fig. 2), while the AT1/AT10 family can be subdivided into two clusters: AT6, 7 and 10 in one group, with a similar profile to AT11/12/13, and the rest of the family into another group of proteins, with a longer N-terminal domain. The presence of 6 to 11 putative transmembrane domains is consistent with the hypothesis of a role as amino acid carriers. The only amino acid transporter whose secondary structure has been studied experimentally is AtAAP1, for which a model was proposed consisting of 11 transmembrane domains (Chang & Bush, 1997).

4.2. AT proteins could not be functionally characterized by expression in yeast.

AT4, AT7, AT11 and AT12 were unable to complement the mutations of the 22Δ9AA strain, as well as those of the JT16 strain. This results were consistent with previous studies as these genes never appeared in previous screenings made by our group. The reason for the failure to complement any yeast mutant strain remains to be established. Several explanations can be given. It can either be concluded that these putative transporters are unable to transport these specific amino acids or at least assumed that the transport is not quantitatively sufficient to complement the yeast mutants. It remains possible that these transporters are specific for a group of amino acids not taken into consideration. The subcellular localization could be other than the plasma membrane as it is originally in the *Arabidopsis* cells and this mistargeting could explain the lack of complementation. The homology to the yeast AVT proteins suggests a possible role as amino acid transporters localized on the tonoplast. This possibility has been thoroughly analyzed in the following section. Finally, another hypothesis is that the

transporters could require additional factors not present in the yeast, such as plant specific kinases or folding chaperones and therefore be inactive in yeast.

4.3. AT proteins are putative vacuolar amino acid transporters.

The AT proteins have been identified *in silico* as the homologs among the *Arabidopsis* membrane proteins (Table 1,2,3) of the ScAVTs, members of the ATF1/AAAP family in yeast. As obvious from the phylogenetic tree of the AAAP family (Fig. 7), the AT proteins are related to ScAVT1 (AT1 to 10) and to ScAVT3 and 4 (AT11,12,13). Therefore, it cannot be excluded that the function of these proteins in *Arabidopsis* is similar to that of the ScAVTs in yeast. Russnak *et al.* (2001) indicated the AVTs as transporters of amino acids with a subcellular localization in the tonoplast. In particular, AVT1 imports large neutral amino acids (Tyr, Ile, Leu, Asn, Gln) into the vacuole, while AVT3 and 4 have the same range of amino acids as transport substrates but are exporters from the vacuole.

Vesicles from vacuoles of *Saccharomyces cerevisiae* were isolated and the uptake of radioactive Gln and Lys was measured. The idea was to repeat the experiment of Russnak *et al.* (2001), extending it to yeast expressing AT4, AT7, AT11, AT12 from *Arabidopsis*, to test if these plant proteins were able to increase the uptake of Gln. As no linear kinetics was observed despite several repetitions under different conditions, it was decided to employ other methods to examine the hypothesis that ATs might be vacuolar transporters. Very recently, Prusty *et al.* (2004) showed that the AVTs are not actually localized on the vacuolar membrane, and that they might function as auxin transporters. This could explain why the experimental setup after Russnak *et al.* (2001) did not yield positive results.

Another method that was developed to indirectly verify the hypothesis of a vacuolar localization of the AT proteins was to grow yeasts in the presence of a toxic analog of an amino acid. The idea was that one of the possible methods by which the yeast can grow on this compound is that the compound is sequestered in the vacuole, via active transport. If one of the AT transporters tested were able to transport the toxic analog into the vacuole, it would be possible to infer that would be able to transport the amino acid itself into the vacuole. The same strategy should, in principle, also identify plasma membrane transporters, as an importer would increase the sensitivity to the compound, while an active export mechanism should decrease the sensitivity.

The sensitivity of the 23344c strain to the glutamine analog DON when expressing AT4, AT7, AT11, AT12 was tested; the growth was not restored after transformation with the expression vector pDR containing the coding sequence for these genes.

It is possible to deduce that DON, and probably Gln, are not transported into the vacuole by these putative transporters, or it could also be that they are transported with low efficiency, which does not allow to confer resistance to the compound. ScAVT1 should be able to transport Gln into the vacuole (Russnak *et al.*, 2001), however ScAVT1 was also unable to modify the sensitivity to DON, therefore it is necessary to consider the possibility that this protein may actually not be a vacuolar transporter, as reported in Prusty *et al.*, 2004.

In addition, a novel yeast strain, G119, was developed to find and characterize new amino acid transporters. The ScAVT1 gene was deleted in the strain and a strategy was developed to target the asparaginase activity of the yeast into the vacuole. A tonoplast transporter of asparagine, such as ScAVT1 is required for the growth of this yeast strain on asparagine as the sole nitrogen source. The asparaginase was transfered into the vacuole via transformation with a plasmid that contains the *asp3* gene under the control of the *cpy* promoter and leader sequence. The cytosolic asparaginase activity of the yeast was lost by the deletion of the *asp1* gene (Fig. 12) and it was not restored when the *asp3* gene was expressed (Fig. 13). It is therefore possible to assume that ASP3 did not remain in cytosol. However an important aspect that weakens the result is that no evidence was produced to support the assumption that ASP3 is functionally expressed and that the subcellular localization is indeed the vacuole. To make sure that deletion of AVT1 is essential for the subcellular localization, a new construct should be prepared in which the same genes as in G119 are knocked out except for the vacuolar transporter ScAVT1. If the deletion of AVT1 is responsible for weak growth of G119 on asparagine as the sole nitrogen source, despite the lack of asparaginase activity in the cytosol, this would be further evidence that AVT1 is a transporter of asparagine into the vacuole and also support the assumption that the asparaginase activity has been successfully transfered into the vacuole. The AT genes were also tested with this system and did not restore growth. However, as there is no definitive evidence that ASP3 is functional, it is not possible to exclude that these transporters actually code for vacuolar transporters of amino acids.

A way to improve the system would be the deletion of *asp5*, which codes for Aspartate Aminotransferase 2. The resulting strain would be aspartate auxotrophic and asparagine could be used as sole source of aspartate, instead of source of nitrogen. In this case the growth of the

strain on asparagine would assure that the vacuolar asparaginase is active and more stringent and efficient screening conditions could be used.

A way to further test the possibility of vacuolar localization of the AT proteins was the construction of GFP fusions. Fusion protein constructs were made for AT1, AT11 and AT12 and two of them were expressed in protoplasts (AT1 and AT12). The fusion proteins are localized in internal membranes of the cell, but not on the vacuolar membrane. The localization of the fusions does not give a definitive answer. The fusions could be arrested in the ER under conditions in which they are expressed due to misfolding. For some proteins, specific receptors are present in the ER that mediate the correct targeting: e.g. SHR3 for amino acid permeases is yeast (Ljungdahl *et al.*, 1992).
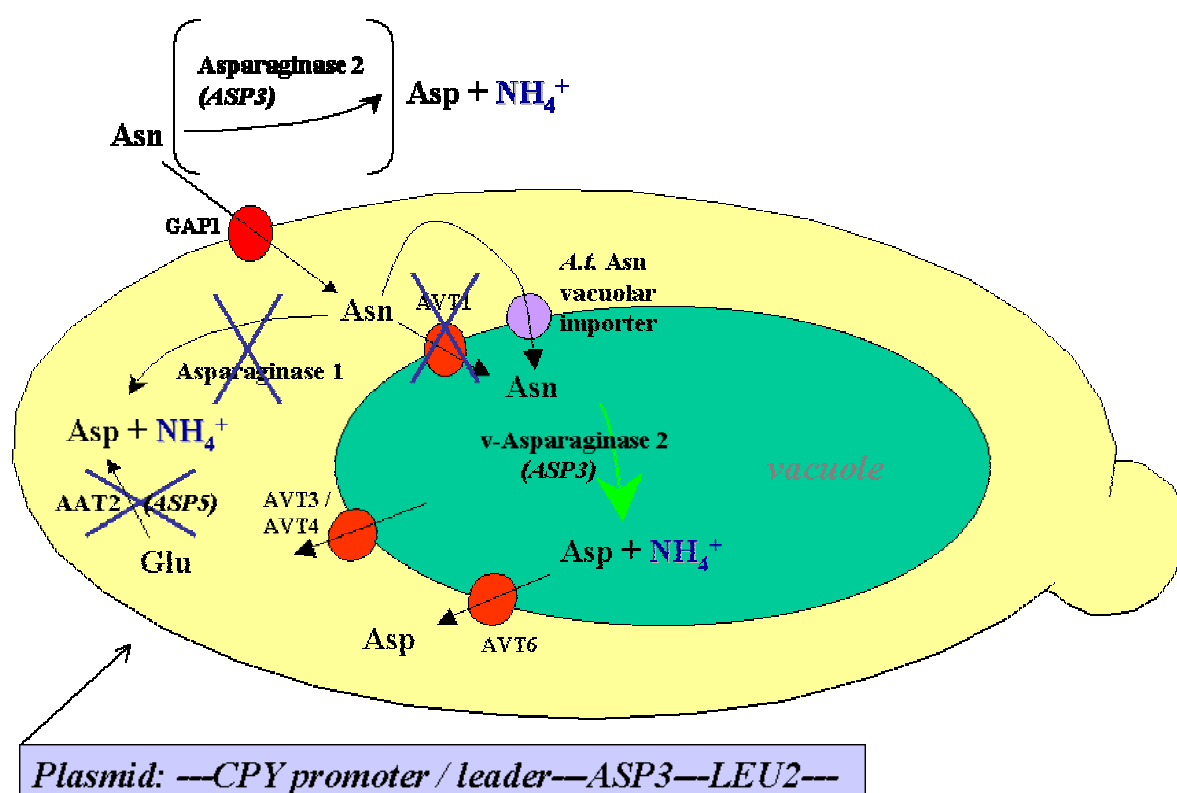

Figure 22 –Possible improvements of the G119 system. A further deletion in the *asp5* gene would make the strain aspartate auxotrophic, so that selection could be made on asparagine as the sole source of aspartate and therefore be more effective.

4.4. AT proteins are putative exporters of amino acids through a vesicle loading mechanism

The export of amino acids from plant cells is a process still unknown at a molecular level. All transporters that have been isolated function as importers of amino acids. However several known physiological processes require the presence of one or multiple exporters of amino acids. Mobilization of amino acid pools from the source organs requires the presence of amino acid exporters, as well as the apoplastic load into the phloem which requires amino acid exporters in the companion cells. The export of amino acids from the sieve elements for transfer into the sink organs requires an export mechanism. Other exporters are required in the xylem parenchyma for the loading of amino acids in the xylem sap.

The subcellular localization of AT1 and AT12 proteins fused to GFP demonstrates that the transporters are localized in the endomembranes in *Arabidopsis* protoplasts. This localization may suggest that these proteins are exporters of amino acids, with a vesicles based mechanism. Amino acids could be imported into these vesicles and after vesicle fusion with the plasma membrane, the amino acids finally get exported into the apoplastic space.

A similar mechanism is proposed for the loading of synaptic vesicles with GABA in mammalians that is mediated by the vesicular transporters VGAT (McIntire *et al.*, 1997). The AT proteins are the closest homologs of the VGAT in *Arabidopsis thaliana*.

A method to isolate and characterize amino acid exporters is the use of amino acid analogs.

A decrease of sensitivity in the yeast strain tested when expressing an amino acid transporter could be an indirect evidence of amino acid export from the cell. One of the most important amino acids that must be exported into the phloem is glutamine, as it is one of the most important amino acids represented in the phloem sap. In this study the use of DON as analog of glutamine did not show a change of sensitivity to this compound in yeast that was expressing the ATs. However, if the transport is mediated by vesicles, the expression in heterologous systems such as yeast might not be functional or the presence of other elements of the plant cell could be necessary for a correct formation and function of the export vesicles.

4.5. AT4, AT5, AT9 and AT12 are differentially expressed in *Arabidopsis*

The promoter activity of AT4, AT5, AT9 and AT12 was studied by expression of GUS under the control of the promoter of these genes. This approach has been successfully used to study in detail the promoter of several plant genes (Martin *et al.*, 1992).

The promoter of AT5 did not show any activity under normal growth conditions, on MS/agar medium or in soil. This leads to the conclusion that AT5 is probably expressed only under particular conditions, like specific stress conditions, that were not identified in this study, or that some other regulatory elements not included in the construct, such as downstream elements present in the introns of the gene, influence the expression. As GUS activity measurements are based on an enzymatic test and dye, detection too minute expression could remain undetected.

The promoter of AT4 shows activity in the hypocotyl region at a very early stage of germination (Fig. 18a). The expression is thereafter confined to the petioles of young leaves (Fig. 18b). This indicates a possible role of this transporter in the supply of amino acids to sink organs, connected to the fast elongation present both in the hypocotyl region and in the petioles of young leaves.

The promoters of AT9 and AT12 show a very similar pattern despite the difference in the length of the regions used in the construct as putative promoter (1973 bp for AT9 and 722 bp for AT12). The promoter of AT12 has a 45,5% similarity with a region between position −820 and position −59 in the AT9 promoter. It is possible that similar elements are present in the two sequences.

The promoters of AT9 and AT12 show promoter activity in young seedlings immediately after germination (Figures 19a and 20a). A role during germination is possible in the remobilization of stored nitrogen from the seedlings. However, the redundancy of the amino acid transporters might explain why the knock-out plants of these transporters did not show any mutant phenotype under the conditions tested. Other transporters, such as AAP1 and AAP2, which are expressed in the vascular system of the cotyledons (Fischer *et al.*, 1995), might have a similar function.

After the germination process GUS activity under AT12 and AT9 promoters is present in leaves, decreasing with the age of the plant and of the leaf (Fig. 20c). This also indicates a possible role of these transporters in the supply of nitrogen of the sink organs. They could work sinergically with AAP6, which is a high affinity transporter responsible of the uptake of amino acids from the xylem (Okumoto *et al.*, 2002).

The activity is also present for both promoters in pollen grains (Fig. 19d and 20d), indicating a role of AT9 and AT12 in the transport of amino acids into heterotrophic tissues during the gametogenesis.

Activity is also present in the two extremities of the silique, indicating a possible role during the supply of nitrogen to the seeds. For AAP1 and AAP2 a function in the development of the siliques has been hypothesized (Fischer *et al.*, 1995), however, other amino acid permeases could also be involved in different steps of the same process.

The clear difference between the activity of the two promoters is the expression in roots, which is only detectable for AT9 in the entire root of young seedlings (Fig. 19a) and in lateral roots in the adult plant (Fig. 19b). The activity in roots can indicate another role in the transfer of amino acids from roots to other organs, or in the uptake of amino acids from the soil. The role of AT9 might be for example auxiliary to the role of AAP3 that is expressed prevalently in roots and might thus be specialized for the transport of root-synthetized amino acids. (Fischer *et al.*, 2002)

4.6. AT7 has an essential role during the gametogenesis

The analysis of T-DNA insertion lines revealed the sterility of the homozygous plants for an insertion in the AT7 gene, indicating an important role of this gene during the gametogenesis. This important result has to be confirmed: the position of the T-DNA insertion is automatically assigned by a large scale sequencing project and should be confirmed by sequencing of the surrounding sequence of the T-DNA insertion. The possibility of a multiple insertion, and that therefore the mutation responsible for the phenotype could be in another gene, could be excluded by backcrossing of the T-DNA line with wild type *Arabidopsis thaliana* or by analysis of other independent T-DNA lines with insertion in the AT7 gene. A list of the currently available T-DNA lines that could be used for this analysis is given in Table 9. Further observation are also necessary to establish if the sterility of this line is due to pollen or maternal sterility. All the other T-DNA analyzed (Table 8) did not show a clear phenotype, however, it is possible that a more detailed analysis could reveal a phenotype under determined conditions.

4.7. Conclusions

Amino acid transport in *Arabidopsis thaliana* has still not been completely characterized at a molecular level. The presence of multiple amino acid carriers and putative amino acid carriers, selected by homology to proteins whose function has been demonstrated, requires further efforts to clarify the role of each transporter in the picture of the physiology of the entire plant.

This is still true even if the amino acid import in the cells has been more and more elucidated and entire families of transporters, like the AAPs (Fischer *et al.*, 2002, Okumoto *et al.*, 2002), have been studied at a very detailed level, with models for the functions of each single transporter of the family. Regarding the intracellular exchanges of amino acids, some transporters have been characterized in the mitochondrial family that mediate transport of amino acids.

However, less or nothing is known regarding other molecules like the carriers that mediate transport in and out the tonoplast and into and out of the chloroplasts and the transporters responsible of the export of amino acids from the cell. In this study we analyzed several previously uncharacterized transporters, considering the possibility of their involvement in one of the missing steps, such as the vacuolar transport of amino acids or the export from the plasma membrane. Definitive evidences to support these models must still be produced and require the development of new tools, such as nanosensors for the *in vivo* visualization of the amino acid. The expression pattern has been analyzed through promoter-GUS fusions and a role for AT4, AT9 and AT12 in the supply of nitrogen to sink tissues like developing leaves can be postulated. In addition a role for AT9 and AT12 during the embryogenesis and the developing of the seeds might also be present. The expression of AT9 in the roots indicates a possible involvement of this transporter in the uptake of amino acids from the soil or in the translocation of amino acids from the root to other tissues. These genes have an individual differential pattern of expression and this might explain the apparent redundancy of proteins that play a similar role, that is amino acid transport.

A way to study the transport specificity is necessary to completely understand the physiological role of these transporters. This encounters difficulties due to the lack of complementation of the yeast mutants that have been used up to now to isolate and characterize amino acid transporters. The development of new strains, with potential for the study of intracellular transport is necessary and the G119 strain described in this study for the

individuation of vacuolar asparagine transporters, although not yet functional, is a starting point for the development of these tools.

Alternative methodologies could also be introduced for the detection of amino acid transport. The color assay that has been developed using the 5-methyl-tryptophane could be improved and used for screening of new tryptophane transporters.

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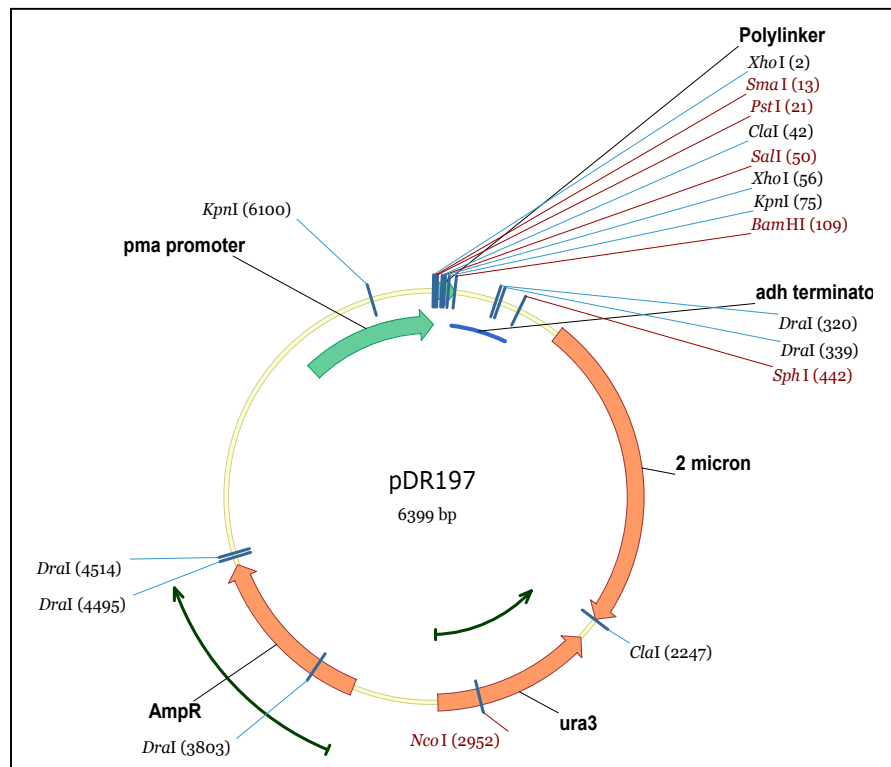
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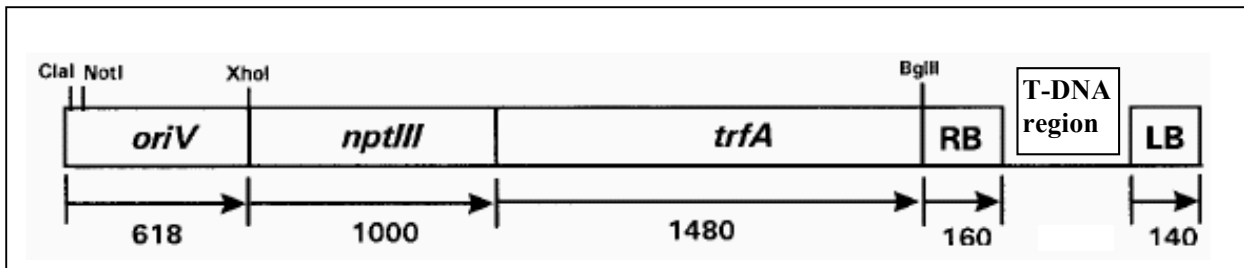**6**   **Appendices**
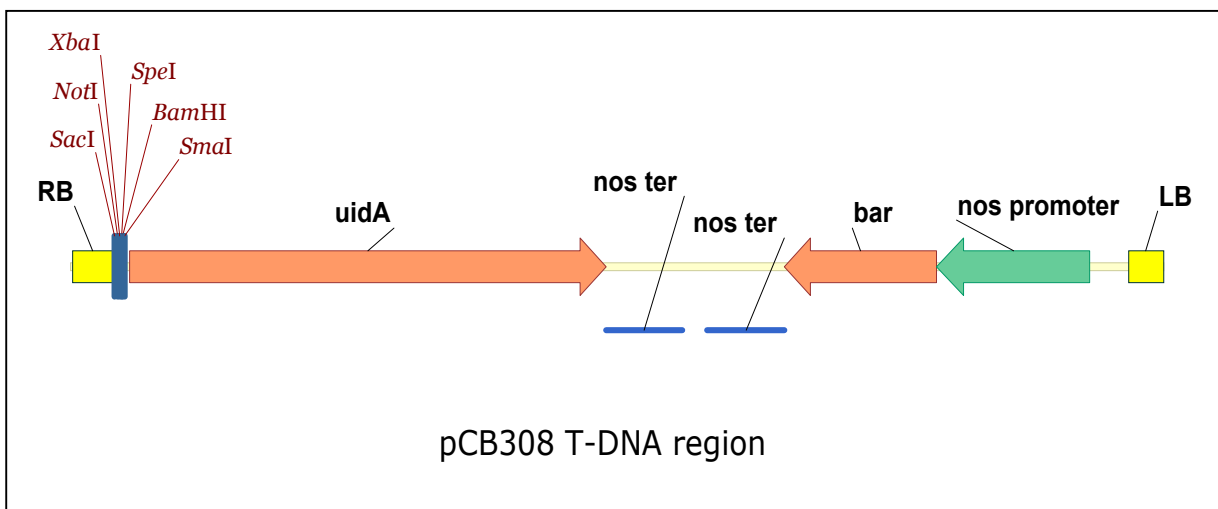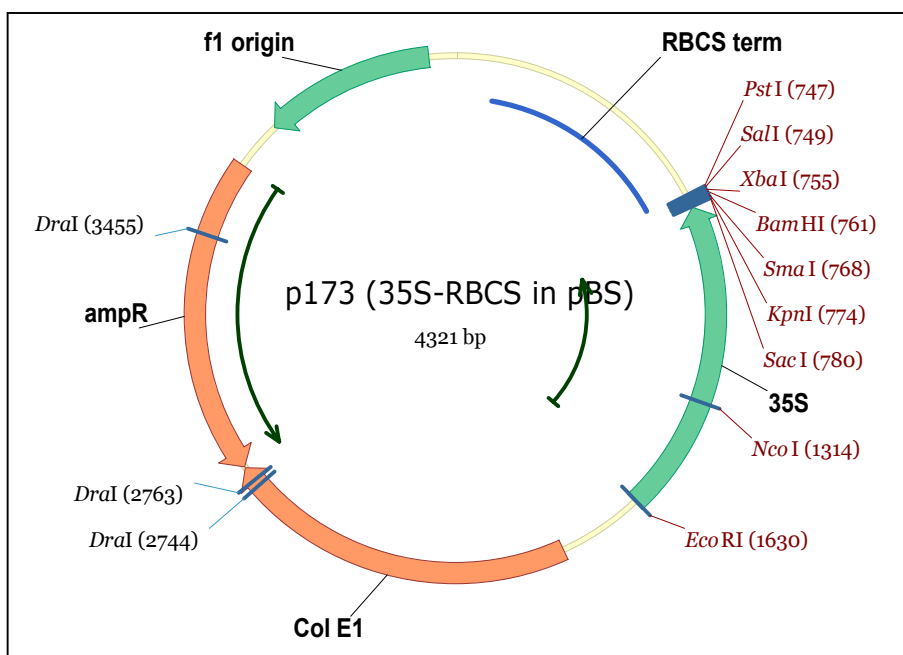
6.1. Vector maps

pDR195
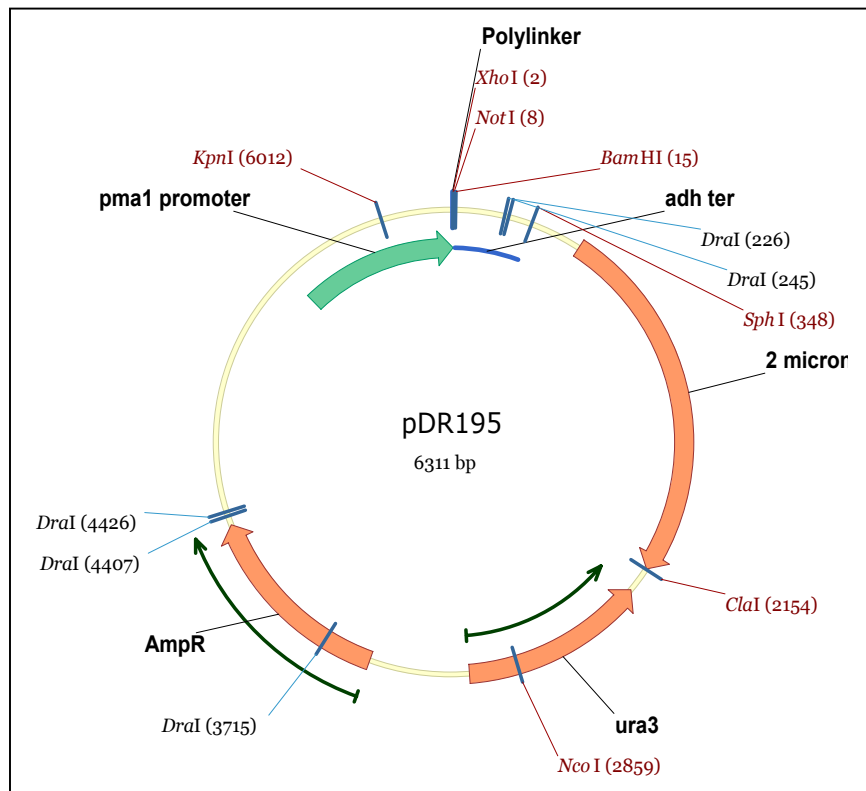
pDR197

pCB308

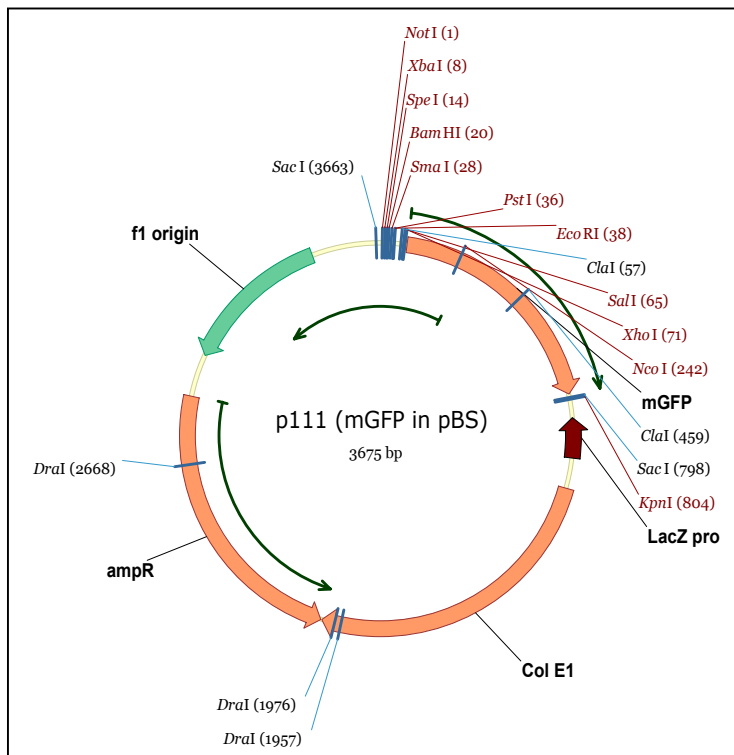(picture modified from Xiang *et al.*, 1999)

pCB308 T-DNA region

pBS-173-35S

p173 (35S-RBCS in pBS)

4321 bp

pBS-110-GFP
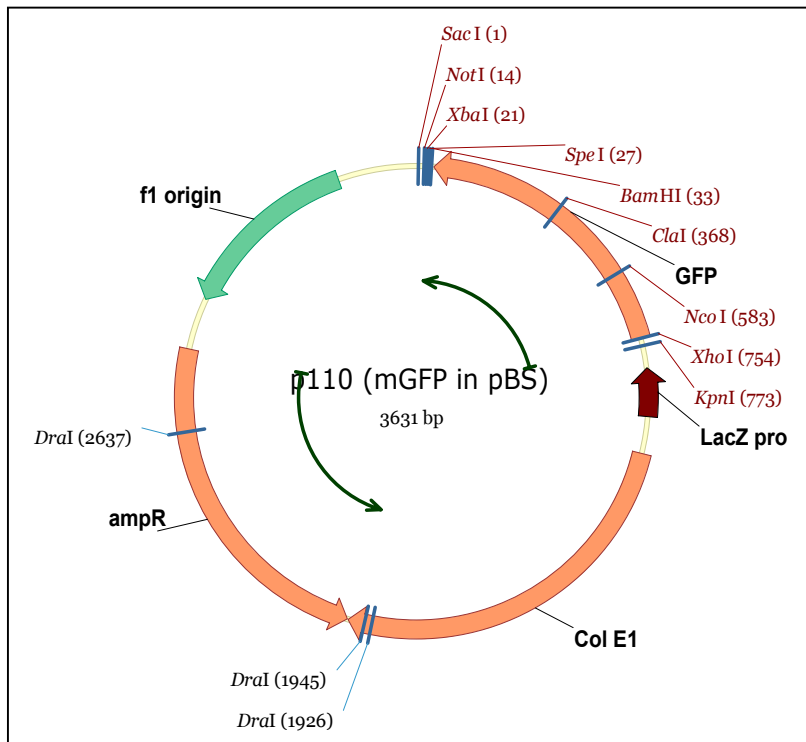
pBS-111-GFP

pCR4Blunt-TOPO (Invitrogen)
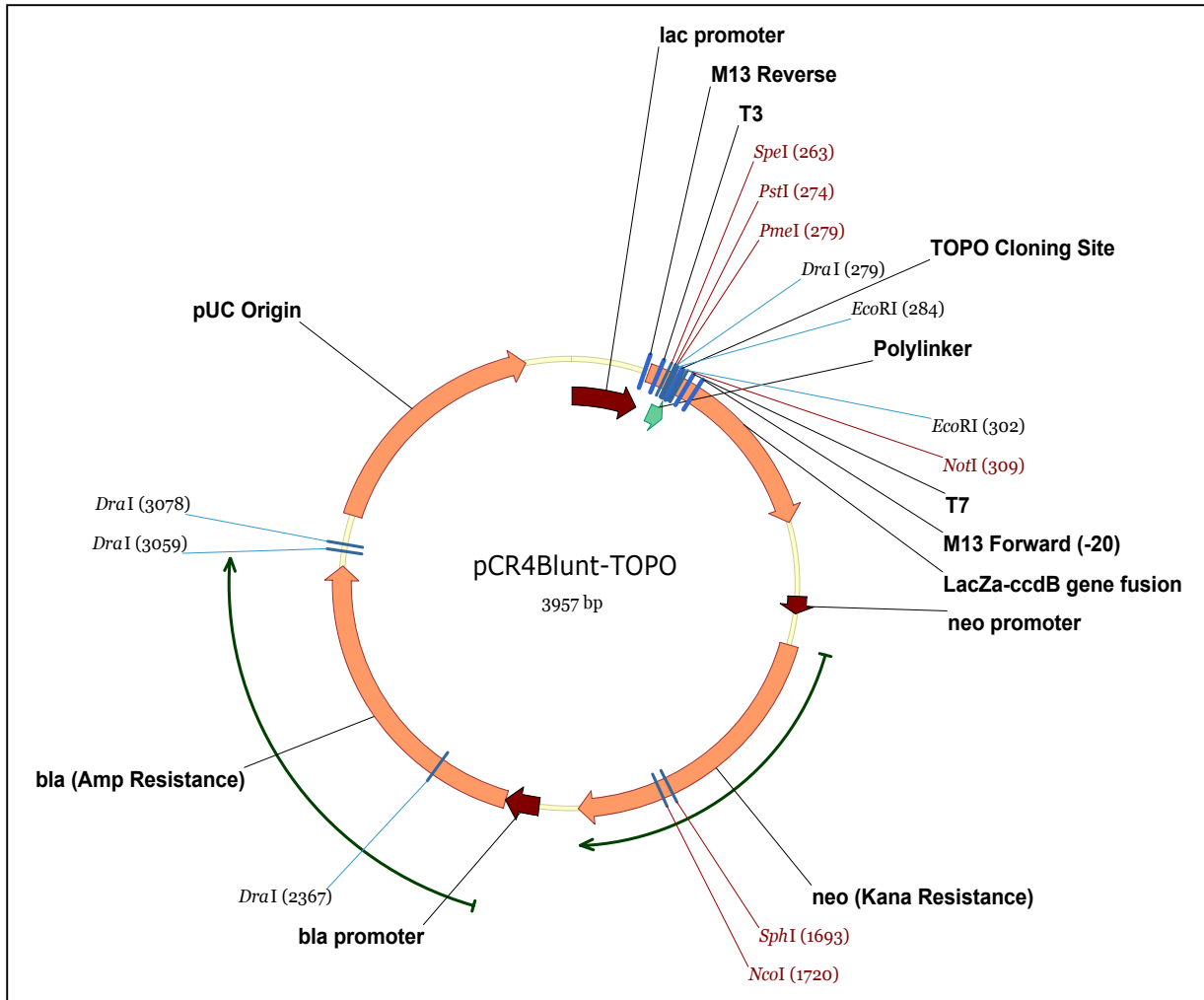
6.2. Composition of complements for yeast media

CSM-ura:

	(mg l^{-1})*
adenine	10
L-Arg HCl	50
L-Asp	80
L-His HCl	20
L-Ile	50
L-Leu	100
L-Lys HCl	50
L-Met	20
L-Phe	50
L-Thr	100
L-Trp	50
L-Tyr	50
L-Val	140

* final concentration in the medium

SC-His-ura:

	(mg l^{-1})*
adenine	21
L-Ala	85.6
L-Arg HCl	85.6
L-Asn	85.6
L-Asp	85.6
L-Cys HCl	85.6
L-Gln	85.6
L-Glu	85.6
L-Gly	85.6
myo-inositol	85.6
L-Ile	85.6
L-Leu	173.4

L-Lys HCl	85.6
L-Met	85.6
PABA	8.6
L-Phe	85.6
L-Pro	85.6
L-Ser	85.6
L-Thr	85.6
L-Trp	85.6
L-Tyr	85.6
L-Val	85.6

* final concentration in the medium

YNB:

	$(mg\ l^{-1})*$
KH_2PO_4	1000
$MgSO_4$	500
NaCl	100
$CaCl_2$	100
biotin	0.002
Ca pantothenate	0.4
folic acid	0.002
inositol	2
niacin	0.4
PABA	0.2
pyridoxine HCl	0.4
riboflavin	0.2
thiamine HCl	0.4
boric acid	0.5
CuSO4	0.04
KI	0.1
$FeCl_3$	0.2
$MnSO_4$	0.4
Na_2MoO_4	0.2

ZnSO$_4$ 0.4

* final concentration in the medium

BA microelements: *

boric acid	16 mM
CuSO4	4 mM
KI	4,6 mM
FeCl$_3$	16 mM
Na$_2$MoO$_4$	4,1 mM
ZnSO$_4$	49 mM

* final concentration in the medium

BA vitamins: *

biotin	10 μM
Ca pantothenate	4,2 mM
inositol	55,5 mM
pyridoxine HCl	4,9 mM
thiamine HCl	3,0 mM

* final concentration in the medium

6.3. Oligonucleotides

6.3.1. Cloning primers for ATs and ScAVT1 cDNAs

```
AT4F:   ACGACGGAATTCATGAAGCAAAACGAGACATTC
AT4R:   ACGACGGGATCCTCAGGTCATTTCGCCGATTAA
AT7F:   AGCAGCCTGCAGATGAGCGAGGACAAAGATTAC
AT7R:   ACGACGGTCGACTCAAAATCGACCAAAAATGTC
AT11F:  ACGACGCTCGAGATGGGTTTTCAGAACGAAGC
AT11R:  ACGACGGGATCCTCACACTTTGACAGAGAAG
AT12F:  ACGACGGAATTCATGAGGTACGATCAGGAAGC
AT12R:  ACGACGGGATCCTCAGGCCTTGGATGTCAGG
AVT1F:  ACAACACTCGAGATGCCTGAGCAAGAACCATTG
AVT1R:  ACAACAGGATCCTTATGAAATAATTGCAGCACC
```

6.3.2. Cloning primers for AT promoters

```
proAT4-F:   AAGGTGCTCAGCTACCAAAATAAAACG
proAT4-R:   GTTTTGGGATCCCTACTTTGTTTGTATTCTTCTACTGTGC
proAT5-F:   ATTGTGATAACTTCCTTCTTCTTG
proAT5-R:   CTTGTCGGATCCATTTCGAACAAACCCAAGTAAG
proAT9-F:   TTGCAGATTTCGAAAGTGAGATAG
proAT9-R:   TACGTGGGATCCTTTTTCACTTTATGAAATCTGATTTG
proAT12-F:  TCTCCTGAGTCTGATTTGAAAAG
proAT12-R:  ATCGTAGGATCCGCCGATCGAAATTGAGAGTG
```

6.3.3. Cloning primers for AT-GFP fusions

```
AT1(GFP)-F:   CTGCAAGGATCCTGATGAAACTCGACGAAGAATT
AT1(GFP)-R:   GTAGATACTAGTCTGCAGTTAATCTGTCATTTGGTTGG
AT11(GFP)-F:  CTGCGAGGATCCTGATGGGTTTTCAGAACGAAGC
AT11(GFP)-R:  GAAGATACTAGTCTGCAGTCACACTTTGACAGAGAAGA
AT12(GFP)-F:  GCAGCATCTAGAATGAGGTACGATCAGGAAGC
AT12(GFP)-R:  CAATAAGTCGACGGCCTTGGATGTCAGGATCT
```

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