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Intracellular vesicle transport in *Arabidopsis thaliana*: Functional characterization of the t-SNARE homologue AtSNAP33

DISSERTATION

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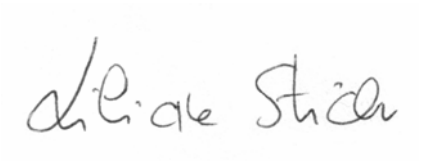

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Abstract

The secretory pathway is a complex endomembrane system essential for all eukaryotic cells. It transports proteins to the extracellular space or to the vacuole. All proteins which are secreted are synthesized at the endoplasmic reticulum (ER) and have a signal peptide in common. The signal peptide is necessary for the transport into the lumen of the ER where the correct folding of the protein takes place. Then proteins are packaged into vesicles which bud of from the ER and transported to the *cis*-Golgi and fuse with it. The proteins go through the Golgi apparatus and at the trans Golgi network (TGN) they are sorted into vesicles destined for several organelles including the prevacuolar compartment (PVC), lytic vacuole, protein storage vacuole, (PSV) or for secretion to the plasma membrane (PM). Transport between the organelles of the secretory pathway occurs by budding of vesicles from a donor membrane and fusion with an acceptor membrane. The fusion of the appropriate vesicles with the correct target membrane is of outmost importance, otherwise the cell will lose its compartmentalisation and collapse.
Soluble *N*-ethylmaleimide-sensitive factor adaptor proteins receptors (SNAREs) and their associated factors were described to be responsible for these fusions steps. The general hypothesis postulates that a membrane associated protein on the vesicle, the v-SNARE, interacts with another membrane associated protein on the target membrane, the t-SNARE. SNAREs are well studied in neuronal cells where vesicles fuse with the plasma membrane to release neurotransmitters after an action potential. In this case the v-SNARE synaptobrevin which is located on the vesicle interacts with the two t-SNAREs SNAP-25 and syntaxin1A at the plasma membrane and form a SNARE core complex which is unusual stable. NSF and α-SNAP two SNARE binding proteins are able to disassociate the complex by hydrolysis of ATP.

In this work an *Arabidopsis* homologue of the t-SNARE SNAP-25, AtSNAP33 was identified as an interactor of KNOLLE. KNOLLE is a cytokinesis-specific syntaxin which is localized at the phragmoplast and is needed for proper cell plate formation during cytokinesis. AtSNAP33 is an ubiquitously expressed membrane-associated protein.
Plants respond to bacterial, fungal and viral pathogen attack by the synthesis of several pathogenesis related (PR) proteins. Some of these PR's are synthesized at

the ER and transported to the extracellular space by the secretory pathway. AtSNAP33 is induced in infected leaves as well after mechanical stimulation such as touch and wind and wounding.

A T-DNA insertion in the AtSNAP33 gene caused loss of AtSNAP33 function, resulting in a lethal dwarf phenotype. Seven days after germination reactive oxygen intermediates (ROI) and lesion formation can be observed on the cotyledons. The lesions spreads out over the whole plant expect the roots and after 3 - 4 weeks the upper green part of the plant is dead. The root growth rate in the mutants was lower compared to the wild type which were grown in the same conditions. In 7 day old *atsnap33* mutants the defence genes PR-1, PR-2, and PDF1.2 were upregulated whereas the two homologues of AtSNAP33, AtSNAP30 and AtSNAP29 were not expressed. *Atsnap33* mutants and Columbia plants as a control were transformed with a barley α-amylase which is constitutively expressed and secreted. In the first 7 days there was no difference in secretion of α-amylase and the mutant expressing α-amylase. But after 7 days the secretion of α-amylase was inhibited in the a*tsnap33* mutant. This correlates exactly with the development of the lesion mimic phenotype. The reduction of the root growth together with inhibited secretion of α-amylase indicate that AtSNAP33 is necessary for successful secretion of proteins.

A yeast two hybrid screen with AtSNAP33 as a bait, led to the isolation of two not yet described syntaxins (t-SNARE) AtSYP122 and AtSYP43. AtSYP122 is constitutively expressed in roots but not in inflorescence stems, leaves, flowers and siliques. However *AtSyp122* transcripts were induced in leaves after inoculation with pathogens. *AtSyp43* transcripts were not expressed in roots, inflorescence stems, leaves, flowers and siliques as analysed by RNA blot hybridisation. Neither pathogen inoculation nor mechanical stimulation led to the induction of the expression of *AtSyp43*. Interestingly, the *AtSyp122* and *AtSyp43* transcripts were highly expressed in *atsnap33* plants which show no expression of AtSNAP33. The biological function and localization of AtSYP122 and AtSYP43 are still unknown.

Zusammenfassung

Alle eukaryontischen Zellen benötigen für die Sekretion ein funktionierendes endomembranes System. Proteine, die sekretiert werden besitzen ein Signalpeptid und werden am Endoplasmatischen Retikulum (ER) synthetisiert. Das Signalpeptid initiiert den Transport in den Lumen des ER und wird danach enzymatisch vom Protein abgetrennt. Korrekt gefaltete Proteine werden in Vesikel gepackt, die sich vom ER abschnüren und mit der *cis* Seite des Golgi Apparats fusionieren. Die Proteine durchlaufen den Golgi Apparat und an der *trans* Seite des Golgis werden die Proteine, die für die Prevakuole, lytische Vakuole oder Proteinvakuole bestimmt sind getrennt von den Proteinen, die ihre Funktion im interzelluläre Raum haben. Der Transport zwischen den einzelnen Organellen, die in der Sekretion involviert sind, findet via Vesikel statt, die sich von einer Geber-Membran abschnüren und mit der entsprechenden Ziel-Membrane fusionieren. Die korrekte Fusion der einzelnen Vesikel mit der entsprechenden Organelle ist von höchster Wichtigkeit, ansonsten verliert die Zelle ihre Kompartimentierung und kollabiert.
Verschiedene SNARE-Proteine (Soluble N-ethylmaleimide-sensitive factor adaptor proteins receptors) und die entsprechenden Faktoren sind verantwortlich für die einzelnen Fusionen von Vesikeln mit den entsprechenden Membranen. Das allgemein akzeptierte Modell postuliert, dass ein Membran assoziertes Protein auf dem Vesikel (v-SNARE) mit einem Membran assozierten Protein auf der entsprechenden Organelle (t-SNARE) wie Schlüssel und Schloss interagieren. SNARE Proteine sind detailliert beschrieben in Nervenzellen. Die Neurotransmitter werden via Vesikelfusion mit der Plasmamembran, beim Eintreffen eines Aktionspotential ausgeschüttet. In diesem Fall interagiert das Synaptobrevin, ein v-SNARE welches auf dem Vesikel lokalisiert ist, mit zwei t-SNAREs SNAP-25 und Syntaxin 1A, beide mit der Plasmamembran verbunden, zu einem ungewöhnlich stabilen SNARE-Komplex. NSF und α-SNAP, zwei SNARE bindende Faktoren sind in der Lange unter Hydrolyse von ATP den Komplex aufzulösen.

In dieser Arbeit wurde ein *Arabidopsis* Homolog von dem t-SNARE SNAP-25, AtSNAP33 isoliert. AtSNAP33 interagiert mit KNOLLE, ein Zytokinese spezifisches Syntaxin, welches an der Plasmamembran lokalisiert ist und essentiell für die

korrekte Bildung der neuen Zellmembran während der Zytokinese ist. AtSNAP33 ist ein allgegenwärtig exprimiertes Membranprotein.
Pflanzen reagieren nach Befall von Mikroorganismen wie Bakterien, Pilze oder Viren mit der Produktion von Abwehrproteinen (Pathogenesis related) PR Proteinen. Einige von den PRs werden am ER synthetisiert und in den interzellularen Raum sekretiert. Infizierte, wie auch mechanisch beanspruchte Blätter von *Arabidopsis* haben eine erhöhte Menge von AtSNAP33 im Vergleich zu nicht behandelten Blätter.

Eine T-DNA Insertion im AtSNAP33 Gen, welches den Verlust von AtSNAP33 bedeutet, resultiert in einem letalen Zwerg-Phänotyp der Pflanze. Sieben Tage nach der Keimung kann die Bildung von ROI (reactive oxygen intermediates) und Nekrosenbildung auf den Kotyledonen beobachtet werden. Die Nekrosen breiten sich auf die ganze Pflanze aus und nach 3 bis 4 Wochen stirbt die *atsnap33* Mutante. Das Wurzelwachstum der Mutante ist reduziert verglichen mit dem Wildtyp, welche unter den gleichen Bedingungen gewachsen sind. In sieben Tag alten *atsnap33* Mutanten sind die Abwehrgene PR-1, PR-2 und PDF1.2 exprimiert im Gegensatz zu den beiden Homologen von AtSNAP33, AtSNAP29 und AtSNAP30, welche nicht exprimiert sind. *Atsnap33* Mutanten wurden mit einer Gerste α-Amylase, welche konstitutiv sekretiert wird, transformiert. In den ersten sieben Tagen war kein Unterschied zwischen der Mutante und Columbia die je mit der gleichen α-Amylase transformiert wurde, festzustellen. Aber in den folgenden Tagen wurde die α-Amylase Sekretion in der *atsnap33* Mutante inhibiert. Diese Inhibition korreliert mit der Bildung der Nekrosen. Die Reduktion des Wurzelwachstums und der Inhibierung der α-Amylase Sekretion in der *atsnap33* Mutante zeigt, dass AtSNAP33 für eine funktionelle Sekretion nötig ist.

Mit Hilfe eines 'Yeast two hybrid Screen' mit AtSNAP33 als Köder, führte zur Isolation von zwei noch nicht beschriebenen Syntaxinen (t-SNARE) AtSYP122 und AtSYP43. AtSYP122 ist konstitutiv in den Wurzeln exprimiert, nicht aber in Stengeln, Blätter, Blüten oder Schoten. Die Transkription von *AtSYP122* wurde durch Inokulation von Krankheitserregern induziert. *AtSYP43* ist weder in Wurzeln, Blätter, Stengeln, Blüten noch in den Schoten exprimiert. Auch das Inokulation von Krankheitserregern in die Blätter stimuliert nicht die Transkription von AtSYP43.

Interessanterweise sind *AtSYP122* und *AtSYP43* in der *atsnap33* Mutante exprimiert, welche keine Expression von AtSNAP33 zeigt. Die biologische Funktion und Lokalisation von AtSYP122 und AtSYP43 sind nicht bekannt.

Abbreviations

aa	Amino acids
Bp	Base pair
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscope
Col	*Arabidopsis thaliana*, ecotype Columbia
DEPC	Diethylpyrocarbonate
DMF	N,N-Dimethylformamid
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylendiamintetraacetic acetate
GFP	Green fluorescent protein
GUS	β-Glucuronidase
IPTG	Isopropyl-1-B-D-thio-1 galactopyranosid
kb	kilobase
KDa	Kilodalton
KEU	KEULE
KN	KNOLLE
Ler	*Arabidopsis thaliana* ecotype Landsberg *erecta*
MOPS	3-(N-Morpholino)-Propansulfonic acid
MS	Murashige and Skoog basal salt mixture
NSF	N-Ethylmaleimide sensitive factor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulfate
SNAP	Soluble NSF-attachment protein
SNARE	SNAP receptor
SNP33	AtSNAP33
Tris	Tris (hydroxymethylate) aminomethane
WS	Arabidopsis thaliana ecotype Wassilewskija
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranosid

GENERAL INTRODUCTION

Cells are the basic units of life. They exist either singly in unicellular organims or in groups in multicellular organisms. Cells are surrounded by a membrane. In addition eukaryotic cells have a number of distinct internal membrane bounded compartments that enclose unique biochemical environments. Without membranes there would not be any life.

There are several different membrane systems in eukaryotic cells and all possess common structural and functional properties. Every membrane is composed of phospholipids. Phospholipids are molecules constructed from glycerol and fatty acids with a hydrophilic head and a lipophilic tail. The amphipathic nature of the lipids allows the arrangement in a bilayer, the lipophilic parts towards each other and the hydrophilic part at the periphery. This bilayer serves as a general permeability barrier for most water-soluble molecules. For a functional membrane proteins and other molecules are associated with the lipid bilayer. There are three different ways in which membrane-bound proteins associate with the bilayer. Peripheral proteins are water-soluble and can be removed by washing, without disruption of the lipid bilayer. In contrast, integral membrane proteins are insoluble because of their hydrophobic (transmembrane) domain and can only be solubilized by destroying the membrane. The third group are the lipid anchored proteins. They behave like integral membrane proteins but they have no transmembrane spanning domain. According to the fluid mosaic model the bilayer is not a fixed structure, in contrary all components are able to diffuse freely within its plane.
All cell membranes are inherited from the mother cell. New membranes can only be produced by growth and fission of existing membranes; and cannot arise *de novo* (Buchanan *et al.*, 2000).

1. The structure of an eukaryotic cell and its organelles

In an eukaryotic cell (Figure 1) the organelles are embedded in the cytosol where about 75% of cell proteins are synthesized. Some of the proteins destined for sectretory pathway, nucleus, peroxisome, chloroplast and mitochondria, contain an amino acid stretch called targeting signal which is not necessary for the function of the proteins but is responsible for the delivery to the correct compartment. When the protein reaches the final destination, in the most cases except the nucleus the targeting signal is cleaved by a peptidase (Buchanan *et al.*, 2000).

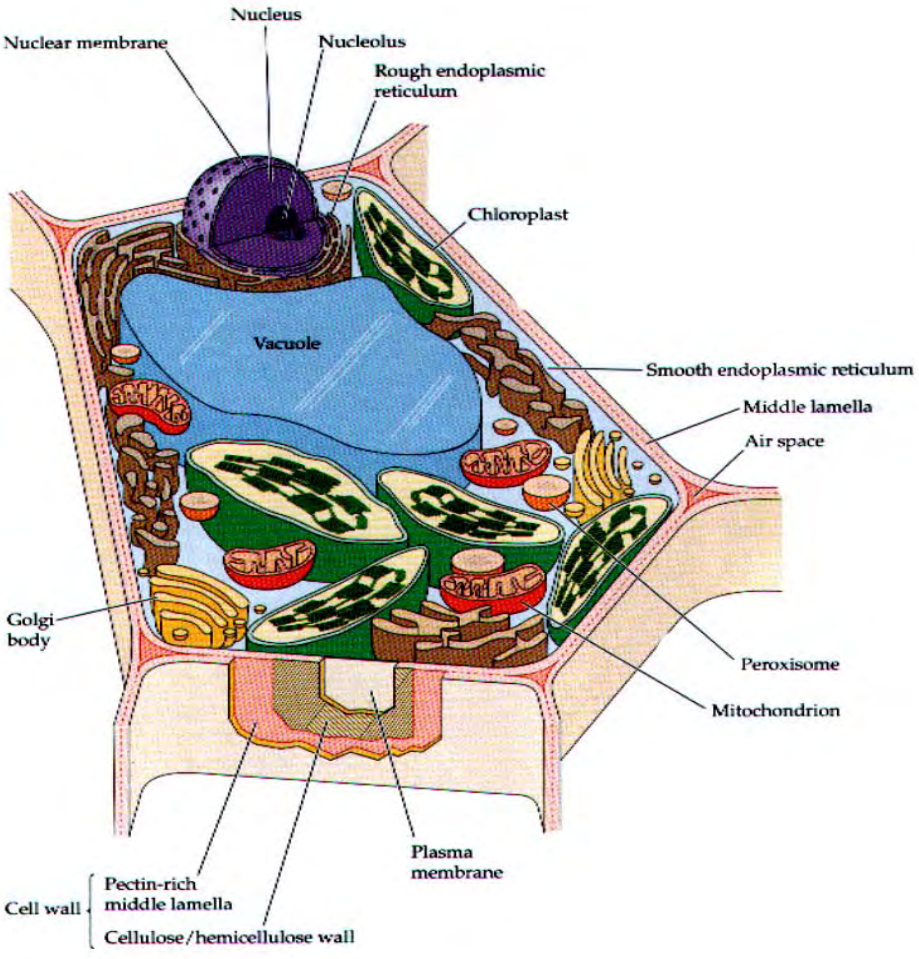

Figure 1: **A scheme of a plant cell with the principal membrane systems**
Reproduction of the mesophyll leaf cell with their organelles (Buchanan et al., 2000).

1.1 Plasma membrane

The plasma membrane is a single bilayer membrane forming the outermost boundary of the living cell and functions as an active interface between the cell and its environment. It is controlling the transport of molecules and transmits signals. The plasma membrane consists of lipids, protein and carbohydrates. The exact composition of the plasma membrane varies in different organisms and different organs. Temperature can also have an influence of the composition of the plasma membrane as was observed in cold acclimation of plants where there is a clear shift from saturated to unsaturated phospholipids (Buchanan *et al.*, 2000).

1.2 The secretory pathway

The secretory pathway is an endomembrane system including the endoplasmic reticulum (ER), Golgi apparatus, endosomes, prevacuolar compartments, vacuole or lysosomes. Proteins and cell wall components are synthesized and processed in the secretory pathway. The secreted proteins are synthesized by ribosomes at the ER. Ribosomes are macromolecules composed of proteins and ribosomal RNA which catalyse the formation of peptide bonds between amino acid residues to form a polypeptide chain. Secreted proteins need a special targeting signal to pass into the lumen of the ER. The polypeptide are folded in the lumen and packaged into vesicles that move to the Golgi. The proteins go through the Golgi and at the *trans*-Golgi network secreted proteins are sorted from vacuolar proteins. Proteins destined for the vacuolar compartment are first delivered by vesicles to the prevacuolar compartment or endosome then to the vacuole. Secreted proteins are transported by vesicles to the plasma membrane where they fuse with it and release their content into the extracellular space. This correspond to the default pathway. The delivery from the ER to the vacuole or plasma membrane is the anterograde pathway in contrary to the retrograde pathway which delivers molecules backwards. (Buchanan et al., 2000) (Figure 2).

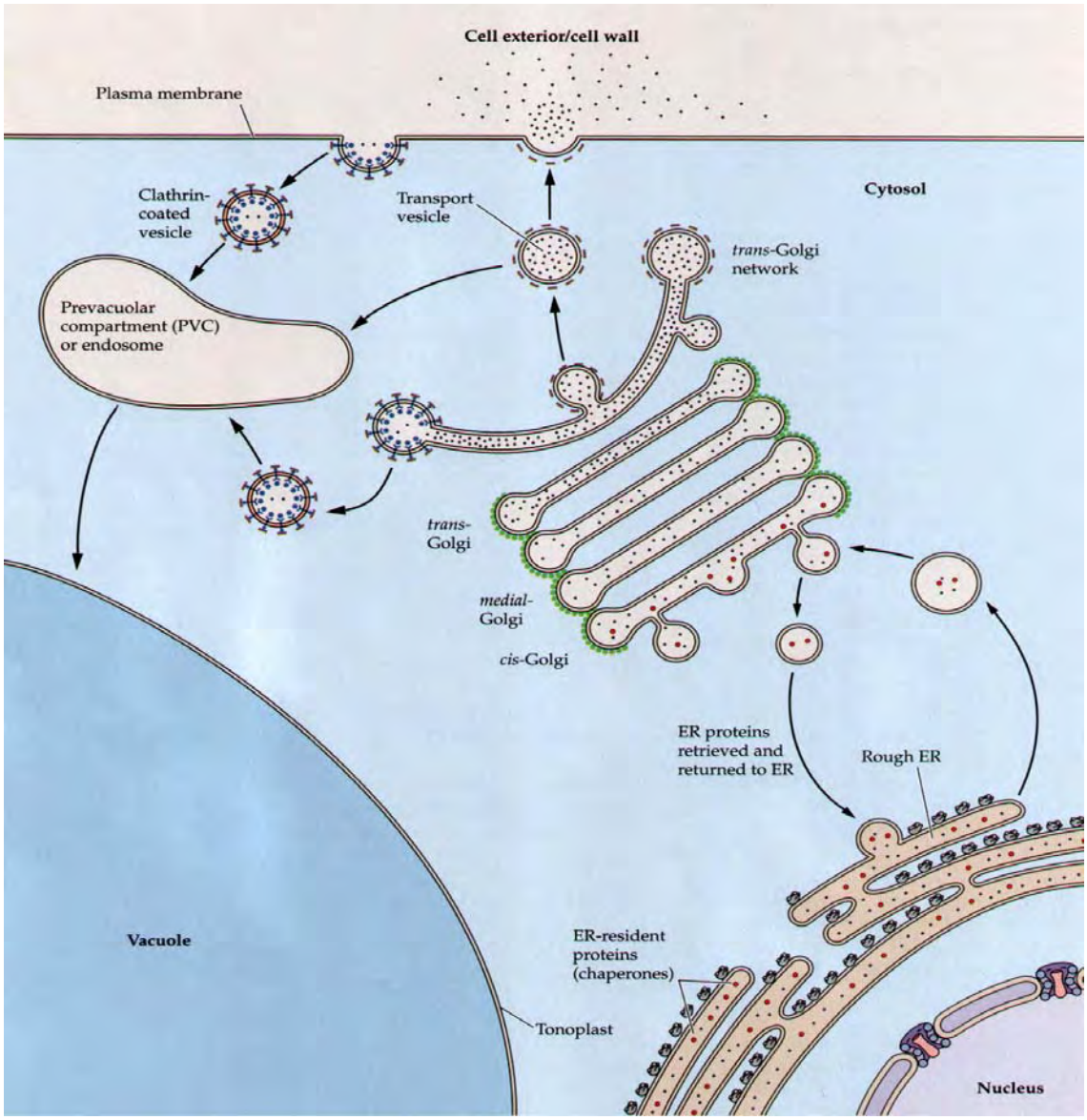

Figure 2: **The secretory pathway for protein synthesis and sorting** Proteins are synthesized and transferred into the lumen of the ER. From there the proteins are transported by vesicles to the *cis*-Golgi. The proteins go through the Golgi and proteins destined to the vacuole are sorted and packaged at the TGN into clathrin coated vesicles. These vesicles are transported to PVC before they reach the vacuole. Proteins determined for exocytosis are transported by vesicles to the plasma membrane where they fuse and release their contents into the extracellular space. Endocytotic vesicles budding off from the plasma membrane are transported to the PVC (Buchanan et al., 2000).

1.3 Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an organelle bounded by a single bilayer membrane and forms a dynamic network of continuous tubules and flattened sacs expanding in the entire cell. Morphologically, mainly three types of ER can be distinguished: the rough, the smooth ER and the ER directly connected with the

nuclear envelope. The function of the rough ER includes the synthesis and processing of proteins. There are ribosomes bound to the surface of the rough ER. (Bar-Peled *et al.*, 1996 and Buchanan *et al.*, 2000).

Proteins which enter the ER have a targeting peptide, the signal peptide which directs them to the ER. The mRNA for such a protein is transcribed in the nucleus. In the cytoplasm it associates with free ribosomal subunits. The signal peptide on the nascent polypeptide chain binds to the signal recognition particle (SRP). The ribosome with the SRP associates with the SRP receptor located on the rough ER and the protein is synthesized and transferred by the translocation apparatus to the lumen of the ER. Then the signal peptide is cleaved by a signal peptidase and the freshly synthesized peptide can be folded correctly. Integral membrane proteins have additional stop-transfer signals, which arrest the translocation, and the appropriate hydrophobic regions are integrated into the membrane to become transmembrane domains. Membrane proteins with more then one transmembrane domain have a second start-transfer signal which reinitiate translocation until the next stop-transfer peptide causes polypeptide release, and so on (Buchanan *et al.*, 2000) (Figure 3).

Some regions of ER lack bound ribosomes and are called smooth ER. The smooth ER makes only a small part of the total ER. In human testis cells the smooth ER can be the place of lipid metabolism or synthesis of steroid hormones from cholesterol (Alberts *et al.*, 1993).

Another function of the ER is glycosylation of proteins. Preformed oligosaccharides are transferred en bloc from dolichol to asparagines residues in the sequence Asn – X – Ser/Thr by a oligosaccharyl transferase (where X is any amino acid except proline) (Buchanan *et al.*, 2000).

The ER is not an isolated organelle, in contrary it has contact nearly with all other organelles. In particular, the ER is the first station in the secretory pathway which transports molecules to the extracellular space and to the vacuole. A very intensive exchange exists between the ER and the Golgi apparatus (Vitale and Denecke, 1999, Mellman and Warren, 2000).

1.4 Golgi apparatus

The Golgi apparatus is a collection of flattened single membrane-bounded cisternae surrounded by small vesicles and is a central part of the secretory pathway. The

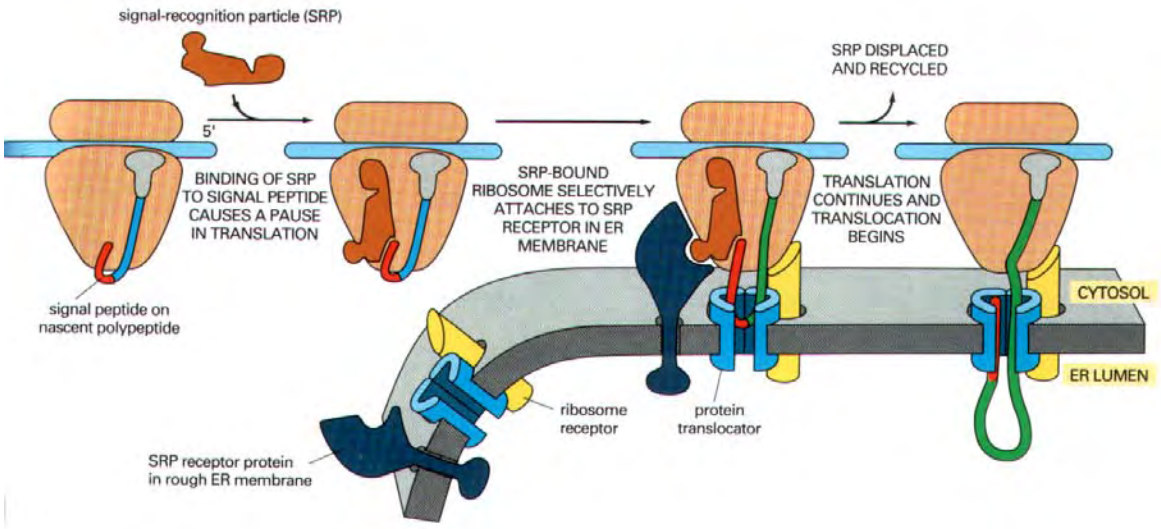

Figure 3: **Translation of a polypeptide at the rough ER** The mRNA transcribed binds to the two subunits of the ribosome in the presence of a signal peptide. SRP binds to it and the whole complex binds to the ER membrane. The translations continues until the polypeptide chain is in the ER lumen. The signal peptide is cleaved by a peptidase (Alberts et al., 1994).

functional unit of the Golgi apparatus is the Golgi stack consisting of several cisternae. The Golgi stack has a distinct morphological polarity with a *cis-, medial-* and *trans*-face and a trans Golgi network (TGN). The number and localisation of the Golgi stack are dependent on the type of cell (Buchanan *et al.*, 2000).

The proteins are transported from the ER to the Golgi by membrane bounded vesicles which fuse with the cis face of the Golgi. The proteins are then transported through the Golgi and glycosylated proteins are modified. Depending on its final destination the protein is packaged into membrane bounded vesicle which fuse with plasma membrane or the membrane of the vacuole (Mellman and Warren, 2000).

In plant cells complex cell wall polysaccharides are synthesized as precursors by enzymes in the Golgi and TGN, except cellulose and callose which are synthesized at the plasma membrane. The cell wall polysaccharides are transported to the plasma membrane by membrane bounded vesicles, there they are released to the extracellular space and are integrated in the existing cell wall (Buchanan *et al.*, 2000).

1.5 Vacuoles and lysosomes

Vacuoles, the most voluminous organelles in most plant cells are bounded by a single membrane, the tonoplast. Lysosomes the corresponding organelles in animal cells are smaller but have the same lytic properties as the vacuole. The presence of hydrolases including proteases and nucleases allow the breakdown and recycling of nearly all cellular components. The vacuoles are large reservoirs of protons and metabolically important ions such as calcium. The control of the release and uptake of these compounds from the cytosol helps to regulate the homeostasis of the cell. The storage of soluble molecules such as ions, sugars and polysaccharides drives the osmotic uptake of water generating the turgor pressure needed for cell enlargement. The vacuole is also used to store large amounts of proteins. A great variety of toxic compounds accumulate in plant vacuoles and help are used for the defence against microbial pathogens and herbivores. Excretion of toxic compounds into the vacuole helps also detoxifying the cell (Marty, 1999).

Soluble protein destined to the vacuole are separated in the TGN from proteins destined to the plasma membrane. Vacuolar proteins require a sorting signal. There are different targeting signals, some vacuolar proteins contain a N-terminal propeptide (NTPP) as a targeting domain. In this NTPP domain four amino acids Asn-Pro-Ile-Arg are necessary and sufficient for sorting to the vacuole (Matsuoka *et al.*, 1995). Other vacuolar proteins contain a C-terminal propeptide (CTPP). In CTPP a sorting consensus was not identified, nevertheless the hydrophobic residues in the CTPP are important for the targeting (Neuhaus, *et al.*, 1994). The NTPP and CTPP are cleaved to give rise to the mature protein. In addition, other vacuolar proteins contain a targeting signal in an exposed region of the mature protein. These targeting signals have to be recognized by a receptor protein on the Golgi. Indeed, a protein of 80 kDa, called BP-80, has been identified in pea. It is an integral membrane protein that is localized in the Golgi complex and in small vacuolar structures (Kirsch *et al.*, 1994). BP-80 binds with high affinity to the NTPP vacuole-targeting signals (Paris *et al.*, 1997). A homologue of BP-80, AtELP (for *Arabidopsis thaliana* epidermal growth factor-like protein) was isolated in *Arabidopsis*. The vacuolar cargo receptor AtELP interacts with the NTPPs of barley aleurain and sporamin from sweet potato but not with the CTPPs of barley lectin (Ahmed *et al.*, 2000).

1.6 Nucleus

The nucleus containing the cell's genetic information of the cell is bounded by a double membrane, the nuclear envelope. The envelope separates the genetic material in the nucleus from the cytoplasm and controls nucleo – cytoplasmic exchanges by nuclear pores. The nuclear pores are the sites for macromolecular movement into and out of the nucleus. During mitosis the nuclear envelope breaks down into vesicles and reassembles around the daughter nuclei. The proteins in the nucleus are synthesized in the cytosol. Nuclear localization signals (NLS) target proteins into this organelle. The position of the NLS varies and the NLS are not cleaved after entry into the nucleus. This allows the protein to re-enter the nucleus after cell division (Buchanan *et al.*, 2000).

1.7 Peroxisome

Peroxisomes are surrounded by a single membrane. In plants their role depends on the organ or tissue in which they occur. In general they play a key role in photorespiration. Peroxisomal proteins, are synthesized in the cytoplasm and are transported posttranslational into the peroxisomes. Proteins destined for the peroxisome contain a peroxisome targeting signal (PTS) which is only needed for the transport and is cleaved immediately after (Buchanan *et al.*, 2000).

1.8 Mitochondria

To maintain the metabolism, the cell needs energy. Mitochondria are the respiratory machinery that generates energy mainly in the form of ATP by way of the citric acid cycle and the associated electron transfer chain. Mitochondria are bounded by two membranes forming an envelope. The inner membrane forms invaginations and contains mostly membrane-associated protein that include both the respiratory electron transfer chain and the ATP synthase. The outer membrane separates the intermembrane space from the cytosolic space. Some of the mitochondrial proteins

are encoded by the mitochondrial DNA and are translated in the mitochondria. But most of the proteins are encoded by the nuclear DNA, are translated in the cytoplasm and are imported posttranslationally into the mitochondria. Typically all mitochondrial protein synthesized in the cytoplasm contain a presequence which forms a N-terminal targeting domain. This domain is recognized by a protein import apparatus and the unfolded protein is transferred into the matrix of the mitochondria. After this transport process the presequence is cleaved and the protein can be folded properly. Transport of proteins into the mitochondrial inner membrane and the intermembrane space requires a supplemental signal (Buchanan *et al.*, 2000).

1.9 Plastids

Plastids are found in plant cells. Like mitochondria, they are bounded by two membranes forming an envelope. All plastids derived from proplastids. Chloroplasts are plastids responsible for the photosynthesis. Amyloplasts are plastids storing starch. Plastids vary in size, shape, content and function and possess a remarkable capacity to differentiate, dedifferentiate and redifferentiate (Sanderfoot and Raikhel, 1999 and Buchanan *et al.*, 2000).

The outer membrane of the envelope separates the chloroplast from the cytosol. The inner membrane surrounds the stroma. The space formed by the two membranes is the intermembrane space. In the stroma there is a third membrane system the thylakoids. Chloroplasts contain their own set of genes which are translated in the organelle. But as in mitochondria, most of the chloroplast proteins are encoded by the nucleus. These proteins are translated in the cytosol as precursors with an N-terminal targeting peptide, the transit peptide. The transit peptide is recognized by an import apparatus. Chloroplast proteins are first transported in the stroma where the transit peptide is cleaved. Then, according to its function, the protein can stay in the stroma or be transported into the lumen of the thylakoids. In this case, a second targeting domain is required (Buchanan *et al.*, 2000).

1.10 Cytoskeleton

The organelles discussed above are embedded in a dynamic filamentous network, the cytoskeleton which provides structure and mobility. The cytoskeleton consists of three types of fibrous polymers, the intermediate filaments, the microtubules and the actin filaments which are the cell's scaffold. This network provides structural stability to the cytoplasm, anchoring proteins and other macromolecules. The cytoskeleton gives cells and cellular components the possibility of move.

Intermediate filaments from animal cells are composed of monomers of different proteins which form an extended α-helix and N- and C-terminal domain at each end. A functional intermediate filament is composed of 8 protofilaments associated laterally. Protofilaments are long chains of polymerized tetramers which consist of two antiparallel associated dimers. Lamin is one example of such an intermediate filament protein which forms the nuclear lamina thought to stabilize the nuclear envelope. The presence of intermediate filaments in plant is not clear. Some homologues of intermediate filament proteins are characterized in plants like the lamin. But the filaments as described in the animal cells are not found in plant cells (Buchanan *et al.*, 2000).

Microtubules are heterodimeric polymers of α- and β- tubulin and actin filaments are polymers of actin. They occur in all eukaryotic cells. Microtubules as well as actin filaments self-assemble into long polymers with the hydrolyses of ATP for actin and GTP for tubulin. The monomers have an asymmetrical structure, assembled to a filament and determine a polarization of the filament (Buchanan *et al.*, 2000)

Different cytoskeletal accessory proteins, the "motor" proteins, interact with actin filaments and microtubules. The motor proteins have in common a head-domain that binds to the cytoskeleton and a tail-domain that binds the cargo. Myosin, the force-producing protein of muscle interacts with the actin filament. Dynein as well as kinesin interact with the microtubuli and move along the filament but in opposite direction. (Buchanan *et al.*, 2000).

Possible cargo are vesicles as described in tip growing cell. In exocytosis of growing pollen tube the vesicles derived from the TGN are in close association with actin filaments. The high rate of expansion at the tip requires a direct delivery of a large quantity of vesicle containing cell wall precursors without interruption. It is also

thought that organelles moving along actin filaments are at the origin of the cytoplasmic streaming in cells of higher plants.

During mitosis the microtubules form the mitotic spindle apparatus initiated in the mitotic spindle poles. They separate the sister chromatids towards the poles during the anaphase of mitosis. After mitosis, cytokinesis occurs which is the separation into two daughter cells. It is different in animal and plant cells. In animal cells a contractile ring of actin filaments initiates the separation in two daughter cells (Buchanan *et al.*, 2000).

In plants after the separation of the chromatin the new cell plate is formed by fusion of Golgi apparatus-derived vesicles which are transported and / or guided by the phragmoplast microtubules to the equatorial region. The vesicles fuse with each other to form a continuous tubular network perpendicular to the microtubules. This network forms a membrane in the middle of the forming cell plate which continuously grows to the periphery and fuses with the existing plasma membrane. (Narcy *et al.*, 2000 and Straight and Field, 2000).

1. 11 Plant cell wall

The plant cell wall is a network of celluloses, pectic substances and proteins. Many cell wall precursors and enzymes are delivered by the secretory pathway. The polymerisation of the cell wall components take place outside of the cell. The cell wall is preventing the burst of the protoplast due to the osmotic pressure and is providing the size and shape of every plant cell.

The exact number of organelles of a cell is dependent on the developmental stage, tissue and cell-function, but all organelles are necessary for a functional cell (Alberts *et al.*, 1994, Bar-Peled *et al.*, 1996 and Buchanan *et al.*, 2000).

2. The secretory pathway requires a well developed system of membrane bounded organelles and specific signal peptides

The secretion pathway is involved in different developmental processes as growth of the plasma membrane or cell wall formation in plant cells. The growth of the plasma membrane occurs by fusion of membrane bounded vesicles deriving from the Golgi apparatus with the existing plasma membrane. In plant cells, the formation of the new cell plate after mitosis depends of the fusion of vesicles. Growth of the cell wall in a growing plant cell needs a continuous production of cell wall precursors and the corresponding enzymes. These components are transported by vesicles and secreted in the extracellular space where the formation of the cell wall takes place (Figure 2).

2.1 The endoplasmic reticulum: the entry point in the secretory pathway

Most proteins destined to the vacuolar compartment or to the extracellular space cannot be transported directly from the cytosol into these compartments. They have to pass through the secretory pathway, an intracellular system of vesicles, organelles vacuole and membranes including the endoplasmic reticulum (ER), Golgi complex, vacuole and plasma membrane. To enter the secretory pathway these proteins need a label or a signal peptide. The translation of the signal peptide on ribosomes attached to the ER causes the entry in the secretory pathway. The N-terminal signal is interchangeable among plant, animal and yeast proteins and is removed by signal peptidase while the nascent polypeptide is emerging into the ER lumen (Vitale and Denecke, 1999 and Buchanan *et al*., 2000).

Once the protein is in the ER, folding and glycolysation takes places before the protein proceeds to its final destination. Molecular chaperones are responsible for correct protein folding and assembly. They include the heat shock protein (HSP) 70 family, the HSP 100 family and the luminal binding protein (BiP). In addition, protein disulfide isomerase (PDI) catalyses the formation of disulfide bridges (Buchanan *et al.*, 2000). Many secretory proteins are N-glycosylated at asparagines residues by the conjugation with a branched oligosaccharide. The multisubunit enzyme

oligosaccharyl transferase transfers the oligosaccharide from a lipid buried in the ER membrane to appropriate Asn residues of nascent polypeptides posttranslationally or as they enter the ER (Vitale and Denecke, 1999).

The ER functions as a quality controller in the secretory pathway. Incompletely or incorrectly folded proteins, such as folding intermediates are retained within the ER in contrast to misfolded proteins which are degraded. The degradation of defective secretory proteins can take place in the cytosol, vacuole or in the ER itself as it has been shown in yeast. In plants it remains to be established where degradation occurs, possible candidates being the same as in yeast. Misfolded proteins are associated with BiP as illustrated by mutated phaseolin, a bean storage protein, unable to form the natural trimer. This proteins is degraded in a process that is insensitive to brefeldin A and heat shock which otherwise inhibit transport to the vacuole. This indicate that also in plant cells the ER can be involved in the degradation process (Vitale and Denecke, 1999). The quality control increases the efficiency of correct structural maturation and avoids delivery of immature or defective secretory proteins.

Cargo proteins which are transported further are packaged into vesicles which bud from the ER. The departure of loaded vesicles from the ER to the Golgi complex is a poorly understood process. Mainly there are two models for the ER export of soluble proteins. The active transport model implicates a sorting receptor that binds to cargo proteins. ER-resident proteins do not bind to the receptor and stay in the ER. The bulk-flow model implicates spontaneous vesicle budding meaning ER residents as well as secreted and vacuolar proteins are packaged in the vesicles. Further work is required before it can be decided which of the two model is correct (Vitale and Denecke, 1999 and Buchanan *et al.*, 2000).

The mechanism for retention of ER-resident proteins is better understood. The C-terminal tetrapeptide KDEL / HDEL and RDEL of soluble ER-resident proteins ensures that these proteins are returned to the ER if they escape. This process is based on a ligand – receptor interaction where the tetrapeptide interacts with a ERD2 receptor (for ER retention defective) in the *cis*-Golgi complex and is transported back to the ER via the retrograde pathway (Lewis *et al.*, 1992 and Phillipson *et al.*, 2001).

Once the secretory proteins and membranes have passed the quality control in the ER they are packaged into vesicles. The formation of vesicles requires proteins from the cytosol to form a coat on the developing vesicle. The COPII coat proteins are

multimeric proteins present on vesicles in the anterograde transport from ER to the Golgi (Robinson *et al.*, 1998 and Nebenführ and Staehelin, 2001). Before the COPII-coated vesicles fuse with the *cis*-face of the Golgi apparatus these coatomers disassociate. Escaped ER proteins are brought back by the retrograde transport from the Golgi to the ER. The vesicle budding off from the Golgi with the destination ER are surrounded by COPI coat proteins which are biochemically different from COPII proteins (Nebenführ and Staehelin, 2001).

2.2 The Golgi apparatus, the branching point in the secretory pathway

The main functions of the Golgi are to modify proteins for example by modification of the glucan side chains, to synthesize cell wall polysaccharides and to package these molecules in vesicular carriers and send them to their next or final destination. While in mammalian cells Golgi stacks are localized next to the nucleus, their position in plant cells generally seems irregular: apparently they move from a location to another where their activity is needed. The number and size of Golgi stacks is very various in plant cells depending on the physiological condition and varies also in the plant kindom. Golgi stacks are polarized structures (Nebenführ and Staehelin, 2001).

How the proteins move through the Golgi from the *cis*- to the *trans*- face is not known in all details. There are two models of intra-Golgi transport. The ‘vesicular shuttle model’ assumes that cisternae are stable units containing a specific set of enzymes. The proteins would be transported through the stack by vesicular shuttles, which travel in the anterograde direction. Escaped ER resident proteins and recycling of Golgi stack resident proteins would be transported in the same way in the retrograde direction. In comparison the ‘cisternal progression-maturation model’ postulates that the cisternae continuously move through the stack. New cisternae are formed on the *cis* side by ER derived vesicles. The newly formed cisternae receives its Golgi stack specific enzymes by retrograde vesicles transport from the next older cisternae and so on down the stacks. The oldest cisternae is fragmented into the trans Golgi network (TGN) (Nebenführ and Staehelin, 2001). Neither the ‘vesicle shuttle model’ nor the ‘cisternal progression-maturation model’ can explain all observed experimental data. So, Pelham and Rothman, (2000) claim that cargo containing

vesicle move up and down the stack, providing a fast flow of cargo across the Golgi. Protein aggregates that are too large for vesicles can still transit the stack by cisternal progression.

2.3 The vacuole

The trans-Golgi network (TGN) is a major branch point in the secretory pathway and is the site of multiple sorting events that separate soluble and membrane proteins for proceeding to the vacuole from those proceeding to the extracellular space. In leguminous plants most of the synthesized proteins enter the secretory system to be delivered to protein storage vacuoles (PSV). These vacuoles appear to arise *de novo*. Most proteins destined to the PSV are transported by electron-dense vesicles deriving from ER bypassing the Golgi (Marty, 1999).
A second mechanism of vacuolar delivery was discovered in endosperm cells of cereal grains. These cells synthesize a storage protein, prolamin, in protein bodies in a separate part of ER bypassing the Golgi apparatus. The protein bodies are incorporated in the vacuole by autophagy (Herman and Larkins, 1999).
Transport to the larger lytic vacuoles of plant cells is mediated by vesicles. They leave the TGN and transport their proteins to the prevacuolar compartment. From there the proteins move to the vacuole by a transport mechanism which is not known in detail, probably by vesicles (Buchanan *et al.*, 2000).

3. Exocytosis is the fusion of membrane bounded vesicle with the plasma membrane

Exocytosis the final step of the default pathway, is the fusion of the secretory vesicle with the plasma membrane and the release of the cargo to the extracellular space. The fusion of vesicles with the plasma membrane can be a process which occurs within milliseconds. For example synapses of neuronal cells contain a vesicle pool that is attached to the membrane. The arrival of an action potential associated with the influx of Ca^{2+} triggers the fusion of the vesicles and the release of

neurotransmitters like acetylcholine within 0.1 – 0.5ms (Jahn and Südhof, 1999). In plant cells this ‘fusion – time ‘ is not determined but in tip growing cells like pollen tubes there is an estimation of the fusion of 6000 vesicles per minutes based on the growing rate (Thiel and Battey, 1998).

The vesicles which bud off the TGN have to be transported or to be guided to the plasma membrane. The common view is that secretory vesicles are delivered to their target membrane by the cytoskeleton. In mammalian cells secretory vesicles visualized with green fluorescent protein, appear to move in all direction within the cell against the cytosolic stream, suggesting that microtubules maintain vesicles in motion to increase the vesicle-membrane contact (Wacker *et al.*, 1997). Although the cytoskeleton provides a mechanism for vesicle delivery, it is unlikely that this mechanism determines where vesicles dock and fuse (Battey *et al.*,1999). Vesicles arriving at the plasma membrane first dock before they fuse with the membrane. Vesicle docking brings the vesicle membrane in close proximity with the plasma membrane and is reversible. Then the formation of a protein scaffold is thought to be necessary for the establishment of a fusion pore. This scaffold draws the vesicle and plasma membrane together and creates a lateral membrane tension which increases membrane curvature. This promotes hemifusion, the fusion of two phospholipid layer facing one another, one from the target membrane with one of the vesicle membrane, before pore formation occurs, resulting in full fusion and the release of the vesicle content into the extracellular space (Thiel and Battey, 1998 and Battey *et al.*, 1999). Pore formation can lead to full fusion or can be followed by closure and vesicle recycling the so-called “kiss and run” process. How this is regulated is not known. In rat chromaffins cells raised concentrations of extracellular calcium ions shift full fusion to the kiss-and-run mechanism (Alés et al., 1999). Another key factor can be the composition of the phospholipid bilayer. Cone-shaped lipids like phosphatidylethanolamine can promote membrane fusion because they favour hemifusion of two close membranes. (Thiel and Battey, 1998) (Figure 4).

The use of electrophysiological methods opens a new area in the understanding of exocytosis. With the patch clamp technique developed by Neher *et al.*, (1982) using chromaffin cells it is possible to measure single vesicle fusion events with the membrane. The plasma membrane is in an electrical sense a capacitor where capacitance is linearly proportional to the surface area of the membrane. A stepwise increase in capacitance can be observed when a single vesicle membrane is inserted

into the plasma membrane during exocytosis and a stepwise decrease occurs when vesicle membrane is retrieved from the surface during endocytosis. The resolution of

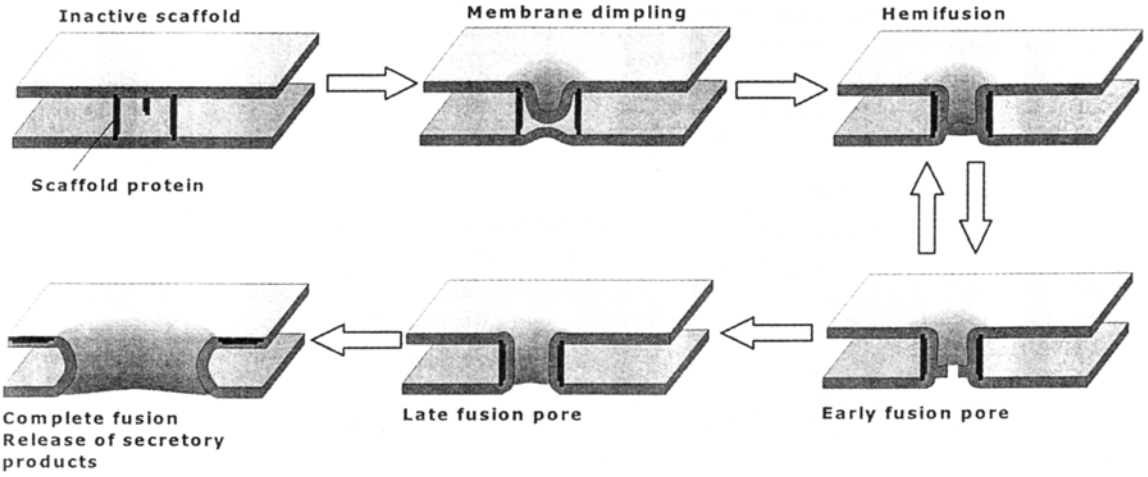

Figure 4: **Vesicle fusion with the target membrane** Two close enough membranes form first a scaffold then the membranes are dimpling and form a temporary stable structure. The early fusion pore is expanding until formation of a complete fusion pore (Thiel and Battey, 1998).

the method allows the recognition of the size of the vesicle which fuses with or is retrieved from the plasma membrane, as well as the determination if a full or a transient fusion took place. The process of vesicle fusion must be highly regulated because if there is only full fusion the plasma membrane will get larger and larger. In contrast, if there is only transient fusion the cell will not be able to grow. There must be a steady state between these two processes (Battey *et al.*, 1999 and Weise *et al*., 2000).

It has been shown that in animal PC12 cells exocytosis events do not take place equally over the entire surface of the plasma membrane. There are cholesterol-dependent clusters, hot spots, that define docking and fusion sites of exocytosis (Lang *et al.*, 2001).

4. Endocytosis

Endocytosis is the reverse process of exocytosis where vesicles bud off the plasma membrane and are transported either to multivesicular bodies (MVB) containing degradative enzymes, partially coated reticulum, endosomes or late Golgi compartments by clathrin coated vesicles. From these organelles, internalised

molecules are transported to the Golgi or vacuole by the retrograde pathway (Battey *et al.*, 1999).

Clathrin coated vesicles are surrounded by an adaptor complex and clathrin triskeleton, a cytosolic protein (Robinson *et al.*, 1998). The formation of this clathrin coat around budding vesicles is necessary to bud off a vesicle and overcome the positive hydrostatic pressure especially in plant cells (Battey *et al.*, 1999).

Receptor mediated endocytosis is an essential process in signal transduction. The receptor-ligand complexes are internalised and processed (Battey *et al.*, 1999).

In nongrowing secretory cells endo- and exocytosis must be well balanced. Electrophysiolocal experiments revealed significant membrane internalisation upon intracellular perfusion with solutions of very low [Ca^{2+}] (Zorec and Tester, 1992). Stomatal movement requires large repetitive changes in cell volumes and changes in surface area. The surface area of guard-cell protoplasts increased under hypoosmotic conditions and decreased after hypertonic treatment (Homann, 1998). The observations in guard cells suggest that endocytosis is implied in membrane recycling which can occur within seconds to minutes.

5. Structure of SNARE proteins / Intracellular fusion reactions

The intracellular organisation of eukaryotic cells is based on the compartmentalization into membrane-bound organelles. Cellular life is dependent on the maintenance of the separation of their unique biochemical environment at all time otherwise the cell would collapse. However, membrane-impermeable macromolecules have to be transported between different organelles without destroying the membrane integrity. One elaborated mechanism occurs in the secretory pathway where membrane-bound vesicles carrying cargo molecules move between the organelles without leakage or disturbance of membranes. Vesicles bud off from one membrane, are targeted to another membrane and fuse with it. But membranes do not fuse spontaneously, they use specialized fusion proteins (Jahn and Südhof, 1999).

Rothman *et al.*, (1994) postulated the SNARE hypothesis to explain this fusion based on the work on neuronal synapses. According to this hypothesis, membrane associated proteins on the vesicle (v-SNAREs) and on the target membrane (t-

SNAREs) interact as key and lock. At every step of the secretory pathway a different set of membrane associated proteins assure the specific fusion of the right vesicles with the right membrane to avoid the loss of compartmentalization. Genetic and biochemical approaches have identified several groups of proteins involved in membrane fusion. These include the SNAREs (SNAP receptors), the associated proteins NSF (N-ethylmaleimide sensitive factor) and SNAPs (soluble NSF attachment proteins) as well as proteins homologous to the yeast Sec1 protein and small GTPases of the Rab family (Jahn and Südhof, 1999 and McNew *et al.*, 2000).

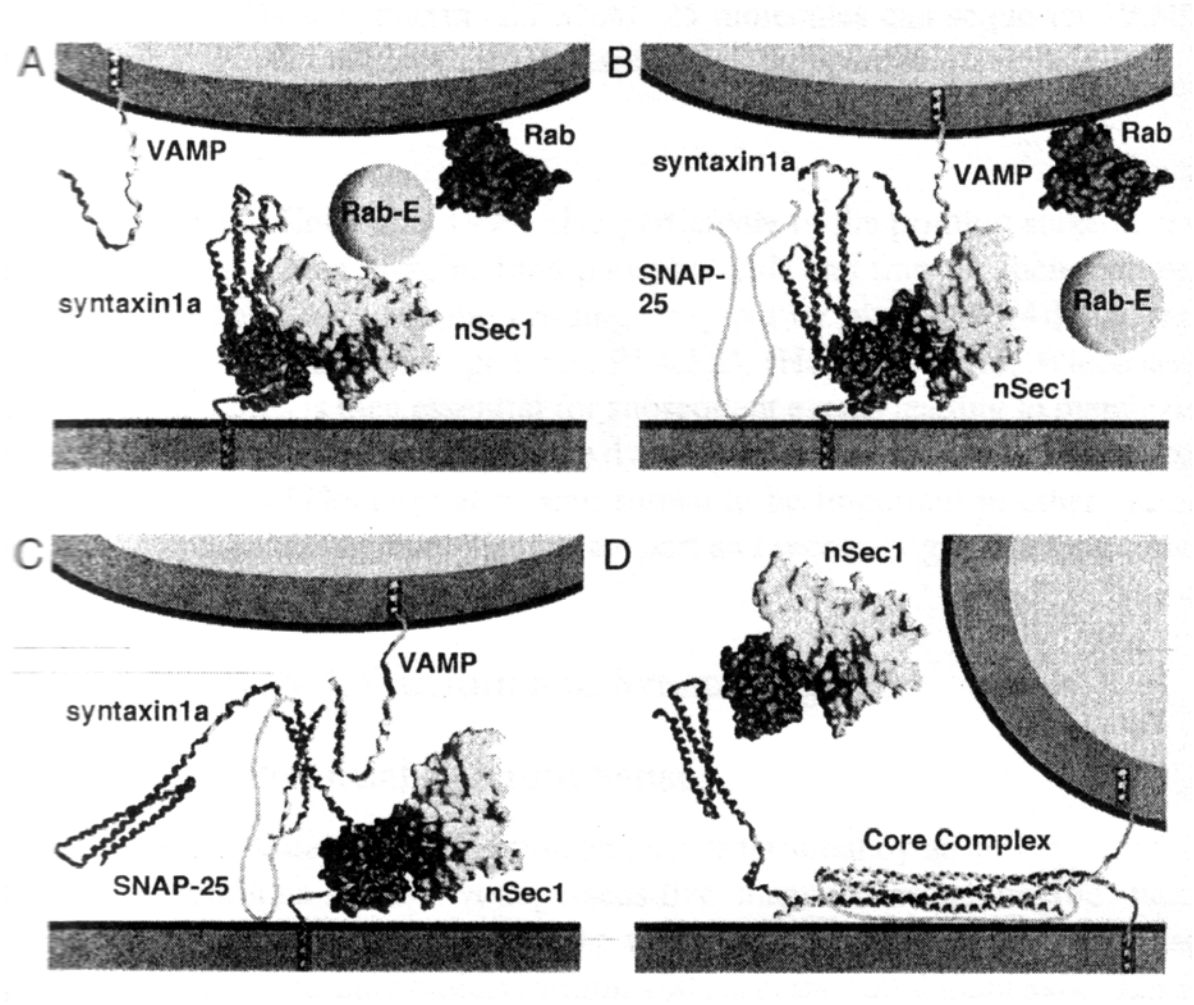

Figure 5: **Model of core complex nucleation** A, syntaxin is bound to nSEC1. B, Rabs cause a conformational change in nSEC1, leading to a destabilization of syntaxin-nSEC1 complex. C, This allows the nucleation of the ternary complex. D, The nucleated complex completes assembly to the SNARE core complex and promotes membrane fusion (Misura *et al.*, 2000).

The family of SNARE proteins independently discovered in neurons and yeast are generally integral membrane proteins associated with either vesicles (v-SNAREs) or target membranes (t-SNAREs). SNAREs are subdivided in 3 groups: the VAMPs

(vesicle associated membrane proteins, v-SNAREs), the syntaxin-like (t-SNAREs) and the SNAP-25-like (t-SNAREs) proteins. In exocytosis at the synapse, one SNARE of each group assembles into a ternary complex with a 1:1:1 stoichiometry (Weber *et al.*, 1998).

Over the time additional SNARE proteins have been discovered which cannot be easily assigned to one of these three groups. Based on sequence analysis, Weimbs *et al.*, (1997) described that all SNAREs share a homologous domain of around 60 amino acids at the N-terminus that is referred to as the SNARE motif. The central residue in the SNARE motif is either an arginine (R) or a glutamine (Q). All known t-SNAREs are Q-SNAREs and most of the v-SNAREs are R-SNAREs (Fasshauer *et al.*, 1998).

The SNARE complex forms an unusual stable structure. *In vitro*, appropriate sets of SNAREs spontaneously form core complexes via their SNARE motifs. The synaptic core complex is formed by four SNARE motifs, one SNARE motif each from syntaxin and synaptobrevin and two from SNAP-25. The crystal structure of the synaptic core complex consists of a twisted four-helix bundle (Figure 5). All chains are aligned in parallel (Sutton *et al.*, 1998). The central layer of the helix bundle is composed of three glutamine (Q) coming from syntaxin and SNAP-25 and one arginine (R) coming from synaptobrevin. Exchange of these amino acids in the corresponding yeast homologues reduced secretion activities in the cells (Ossig *et al.*, 2000). The polar amino acids in the central layer are essential for normal function of SNAREs in membrane fusion (Jahn and Südhof, 1999 and Ossig *et al.*, 2000). This complex cannot be denatured by heat, by the detergent SDS or the protease trypsin (Poirier *et al.*, 1998) and is resistant to proteolysis by botulinum and tetanus neurotoxins (Hayashi *et al.*, 1994).

For a successful fusion of a synaptic vesicle with the target membrane the following SNARE mechanism is postulated. The vesicles bearing the v-SNARE synaptobrevin are targeted close to the appropriate target membrane. The docking of the vesicles is reversible as long as the SNAREs are not primed (Thiel and Battey, 1998). At the target membrane the syntaxin is probably forming a complex with a protein from the Sec1 family. Once Sec1 is released from the syntaxin by small GTPases of the Rab family, the syntaxin undergoes a conformational change and is able to interact with the t-SNARE SNAP-25. Then, the t-SNAREs form a *trans* SNARE complex with

synaptobrevin i.e. they stay on opposite membranes. The SNARE complex brings the vesicle and target membrane closer together and the formation of the fusion pore takes place by an unknown mechanism. The fusion pore expands and the content of the vesicle is released. After the fusion all SNAREs are in the *cis* SNARE configuration in which they are all on the same membrane. The disassociation of this very stable SNARE complex is then performed by the cytosolic factors NSF, an ATPase, and α-SNAP in an ATP dependent process. Little is known about the conformations of the SNARE proteins after disassociation (Jahn and Südhof, 1999 and Misura *et al.*, 2000).

The disassembly of the SNARE complex needs specialized chaperones. SNAP in conjunction with NSF is able to dissociate the stable *cis*-SNARE core complex with ATP hydrolysis (Littleton *et al.*, 2001). NSF belongs to the superfamily of 'ATPases associated with various cellular activities' (AAA protein), has a hexagonal pinwheel shape and binds to the SNARE core complex as a hexamer. As far as known NSF operates on all cis-SNARE complexes. The homologue of NSF in yeast is SEC18 (Jahn and Südhof, 1999).

Three variants of SNAPs exist in mammals referred to as α-, β- and γ-SNAP. SNAPs need to bind to the SNARE complex and stimulates the ATPases acitvity of NSF, before NSF binds. β-SNAP is specific for the brain whereas α- and γ-SNAP are widely expressed. SEC17 is the homologue of α-SNAP in yeast and can functionally substitute the mammalian protein (Woodman, 1997).

The nucleation and disassembly of the SNAREs is well described in the case of the synapse. Based on these results, it is postulated that this is also the case for all SNARE core complex formation in the secretory pathway.

Several studies have shown that SNARE proteins alone probably are not responsible for the targeting but for the fusion of the vesicle with the corresponding target membrane. In a set of experiments (Yang *et al.*, 1999), five different VAMPs, three SNAP-25s, and three syntaxins were tested *in vitro* for their ability to form complexes. Of the 21 combinations tested, all form stable complexes. These results have been confirmed by a similar assay done by Fasshauer *et al.*, (1999). Based on these result they conclude that SNAREs themselves are not essential for the specificity of vesicle targeting but catalyse the fusion by forming the SNARE core complex. These experiments were done *in vitro* and in solution, but recent

experiments with yeast SNAREs anchored in liposomes showed that their interactions can be highly selective (Pelham, 2001).

SNARE complexes are needed for all fusion reactions. There are some reports which show fusion in the absence of SNAREs. Deletion of synaptobrevin in *Drosophila* resulted in a reduction of vesicle fusion, but spontaneous fusion events were still present. Yeast cells lacking the synaptobrevin homologous are heavily sick but still viable (Jahn and Südhof, 1999). Peters *et al.*, (2001) described that the terminal process between docking and bilayer fusion involves an additional non-SNARE protein: subunit V0, a membrane-integral sector of the vacuolar H+ ATPase. V0 subunit located in both opposing membranes can form a complex. The maintenance of this complex and completion of fusion of the membranes are independent of SNARE proteins. But it is not excluded that the SNARE proteins are necessary before V0 mediated fusion occurs.

6. t-SNAREs and v-SNAREs and their homologues in animal cells

The t-SNARE family is subdivided in two groups, the syntaxin-like proteins with at least twenty members in mammalian cells and the SNAP-25 like proteins with four members. Both of them belong to the Q-SNARE according to the classification of Fasshauer *et al.*, (1998).

Syntaxin-like proteins have at C-terminal transmembrane domain which makes them integral membrane proteins. The SNARE motif is located in front of the transmembrane domain, interacts with the other SNAREs and extends into the cytosol. The three dimensional structure of the neuronal syntaxin 1 revealed that there are 3 coiled coils in the N-terminal part of the protein. Coiled-coil domains are α-helical stretches with the possibility to interact with other proteins (Lupas *et al.*, 1991). In the case of syntaxin1 a deep groove is formed between the second and the third helix. According to the authors (Jahn and Südhof, 1999) this groove could be an additional binding site indicating that these proteins have more functions than forming SNARE core complexes. Studies on syntaxin 1 and the corresponding yeast homologue Sso1p showed that the N-terminal domain interferes with the core complex and stabilizes it. Alternatively the N-terminal domain could bind to other

TABLE 1 SNARE proteins in animal cells adapted from Jahn (1999)

Protein	Type	Predominant localization	Genbank accession Nr	References
syntaxin 1A/B	Q	plasma membrane	MM, D45208 MM, D29743	Jagadish *et al.*, 1997, Bennett *et al.*, 1992, Inoue *et al.*, 1992 Bennet *et al.*, 1993
syntaxin 2	Q	plasma membrane	RN, L20823U, L20889U, L20888	Hirai *et al.*, 1992
syntaxin 3	Q	transport vesicles, plasma membrane	RN, L20820	Bennett *et al.*, 1993, Ibaraki *et al.*, 1995
syntaxin 4	Q	plasma membrane	RN, L20821	Bennett *et al.*, 1993
syntaxin 5	Q	Golgi, COP-coated vesicles, ER	RN, L20822, RN U87971	Bennett *et al.*, 1993, Hui *et al.*, 1997
syntaxin 6	Q	TGN, endosome	RN, U56815	Bock *et al.*, 1996, Bock *et al.*, 1997
syntaxin 7	Q	Golgi, lysosome ?	AF031430, AF056323	Wang *et al.*, 1997, Bock *et al.*, 1997
syntaxin 8	Q	ER, endosome ?	AF033109	Bock *et al.*, 1997, Wong *et al.*, 1998, Steegmaier et al., 1998
syntaxin 10	Q	TGN		Bock *et al.*, 1997, Tang *et al.*, 1998b
syntaxin 11	Q		HS, AF071504, AF044309	Tang *et al.*, 1998a, Bock *et al.*, 1996, Advani *et al.*, 1998
syntaxin 12	Q	Endosome	RN, AF035632	Bock *et al.*, 1997, Advani *et al.*, 1998, Tang *et al.*, 1998c
syntaxin 13	Q		AF044581	
syntaxin 16 A/B/C	Q	Golgi	HS, AF008935, AF008936, AF008937	Bock *et al.*, 1997, Simonsen *et al.*, 1998
syntaxin 17	Q			Steegmaier *et al.*, 1998
Hsyn 16	Q		HS, AF038897	Tang *et al.*, 1998d
rbet1	Q	Golgi, COP-coated vesicle	RN, U42755	Hay *et al.*, 1996, Hay *et al.*, 1998
GS15	Q	Golgi	RN, AF003998	Xu *et al.*, 1997
GOS32	Q		RN, AF035822	Wong *et al.*, 1999
GOS28	Q	Golgi, COP-coated vesicle	RN, U49099	Hay *et al.*, 1997, Hay *et al.*, 1998, Nagahama *et al.*, 1996, Subramaniam *et al.*, 1996
membrin	T	ER, Golgi, COP-coated vesicle	RN, U91539	Hay *et al.*, 1997, Lowe *et al.*, 1997
SNAP-25	2Q	plasma membrane	RN, U56261, U56262	Oyler *et al.*, 1989
SNAP-23	2Q	plasma membrane	MM, U73143, HS, Y09568	Ravichandran *et al.*, 1996, Mollinedo *et al.*, 1997, Wang *et al.*, 1997
SNAP-29	2Q	ubiquitous		Steegmaier *et al.*, 1998
vti1b	QT		MM, AF035823	von Mollard *et al.*, 1998, Xu *et al.*, 1998
synaptobrevin 1	R	synaptic vesicle, clathrin coated vesicle	RN, U74621	Elferink *et al.*, 1989, Trimble *et al.*, 1988, Baumert *et al.*, 1989, Mandic *et al.*, 1997
synaptobrevin 2	R	synaptic vesicle, clathrin coated vesicle	RN, M24105	Elferink *et al.*, 1989
cellubrevin	R	microvesicle, clathrin coated vesicle	RN, S63830	McMahon *et al.*, 1993
VAMP 4	R		MM, AF061516	Bock *et al.*, 1997, Advani *et al.*, 1998
VAMP5/6	R	plasma membrane, intravesicular structure	EST clones, AA050010, AA030509, AA222692	Bock *et al.*, 1997
Ti-VAMP	R		MM, X96737	Bock *et al.*, 1997, D'Esposito *et al.*, 1996, Galli *et al.*, 1998
Endobrevin	R	Endosome	MM, AF053724, AF045661	Bock *et al.*, 1997, Advani *et al.*, 1998, Wong *et al.*, 1998
Tomosyn	R		RN, U92072	Masuda *et al.*, 1998
Msec22b	R	ER	MM, U91538	Paek *et al.*, 1997

proteins essential for synaptic vesicle exocytosis like Munc proteins (Jahn and Südhof, 1999)

Syntaxins in mammals are localized to nearly all intracellular membranes (for a review see Jahn and Südhof, (1999) (Table 1). The mammalian syntaxin 1, 2, 3, 4 and the yeast syntaxin Sso1p and Ssc2p are localized at the plasma membrane. The sequence of their N-terminal domains shows a higher similarity than the N-terminal domains of all other syntaxins analysed. These similarities suggest that this domain could have a conserved function specific for exocytosis (Weimbs *et al*., 1997 and Jahn and Südhof, 1999).

The mammalian syntaxin 1a which is located at the plasma membrane forms a complex (Misura *et al.*, 2000) with a cytosolic protein homologous to the yeast Sec1 the nSec1 (or also known as rbSec1 or munc18-a according to the authors (Pevsner *et al.*, 1994, Garcia *et al.*, 1994, Hata *et al.*, 1993). nSec1 is suggested to be a kind of negative regulator that operates upstream of SNARE assembly and controls the availability of syntaxins. However, this hypothesis does not explain why nSec1 is essential for vesicle fusion which was shown by the disruption of the nSec1 ortholog in *Drosophila* (Jahn, 2000). Rabs small GTPases are able to dissociate the syntaxin1a/nSec1 complex before SNARE core complex formation (Hanson, 2000).

SNAP-25 (synaptosomal associated protein of 25 kDa) belongs to the t-SNARE family or according to the terminology used to the Q-SNARE group. It is located to the plasma membrane. SNAP-25 is composed of 206 amino acids and has two coiled-coil domains at the N-terminal end and one coiled-coil domain at the C-terminal end. Crystal structure of the synaptic core complex revealed that the two SNARE motifs of SNAP-25 one found at the N-terminal and one at the C-terminal end are necessary for the tethering with the SNARE motif of the corresponding SNARE partners to form the SNARE core complex (Jahn and Südhof, 1999). A cysteine-rich domain is located between the two SNARE motifs. Palmitoylation of cysteine residues in this domain can provide a membrane anchor in the absence of an enzyme, but in presence of syntaxin it is increased 100-fold (Veit *et al.*, 2000). As long as the syntaxin is present, the cysteine residues of SNAP-25 are not required for membrane localisation (Washbourne *et al.*, 2001).

A single amino acid change of the Leu 203 to alanine or glutamic acid reduced the number of fused vesicles with the plasma membrane in chromaffin cells. Thus Leu

203 at the C-terminus seems to be essential for the function of SNAP-25 in exocytosis (Criado *et al.*, 1999).

SNAP-25 is probably synthesized in the cytosol since it lacks a signal peptide. Like syntaxin and VAMP, SNAP-25 is post-translationally inserted into membranes in the early secretory pathway (Vogel *et al.*, 2000). Vogel *et al.*, (2000) postulated the following insertion model: SNAP-25 assembles with syntaxin which is also translated in the cytosol. The heterodimer is targeted to the membrane by the presence of the transmembrane domain of the syntaxin. At the membrane, SNAP-25 is palmitoylated and even after dissociation of syntaxin it stays attached to the membrane. Associated to the membrane, syntaxin and SNAP-25 are transported to their site of action by the secretory pathway. This was shown by treatment of PC12 cells with brefeldin A, an inhibitor of the secretory pathway. The transport to the plasma membrane was prevented compared to non-treated cells (Gonzalo and Linder, 1998). Syndet and SNAP-23 are homologues of SNAP-25 and are ubiquitously expressed in mammals. Their N-terminal coiled coil is shorter and they are thought to mediate constitutive exocytosis at the plasma membrane (Wang *et al.*, 1997, Wong *et al.*, 1998). SNAP-29 is another homologue of SNAP-25 which is localized on multiple membranes, does not contain a predicted membrane anchor domain (Steegmaier *et al.*, 1998) and is capable of binding to a broad range of syntaxins (Hohenstein *et al.*, 2001).

Mechanically induced plasma membrane disruption leads to an accumulation of vesicles of a various sizes surrounding the disruption site (Miyake and McNeil, 1995). The use of the membrane specific dye FM1-43 revealed that disruption induces Ca^{2+}-dependent exocytosis only around the disruption. Is suggested that the cell size increases after injury. In urchin eggs and mammalian fibroblasts the resealing process after disruption of the plasma membrane can be inhibited by injection of antibodies to kinesin and calmodulin kinase as well as specific inhibitors of synaptobrevin and SNAP-25. Vesicle docking and fusion, mediated by synaptobrevin and SNAP-25 together with other proteins, is needed for resealing of the plasma membrane after disruption (Miyake and McNeil, 1995).

V-SNAREs are R-SNAREs, and consist only of a short proline-rich N-terminal sequence, a C-terminal transmembrane domain and a SNARE motif close to the transmembrane domain and (Lin and Scheller, 2000; Schoch *et al.*, 2001). Most of them are located in the membrane of vesicles. Mammalian synaptobrevin 1 and 2 are

localized on synaptic vesicles and contribute directly to membrane fusion. Synaptobrevin VAMP2 and 3 are localized to clathrin-coated vesicles. VAMP5 and 6 are localized to the plasma membrane which is not typically for v-SNARE (Bock and Scheller, 1997). VAMP8 is localized at the endosomes (Bock and Scheller, 1997). Synaptobrevin (also called VAMP), and its homologues from neuroendocrine PC12 cells are synthesized in the cytoplasm and posttranslational incorporated in the membrane of the endoplasmic reticulum and then transported via the Golgi apparatus to corresponding membrane (Kutay *et al.*, 1995).

7. SNARE proteins are target for clostridial neurotoxins

The Gram positive bacteria of the genus *Clostridium*, are producing the most powerful neurotoxins, the tetanus and botulinum neurotoxins. These toxins are released by the bacteria and selectively attack the nervous system, acting on the SNARE proteins. The tetanus toxin (TeNT), and the seven serologically distinct types of botulinum toxins (BoNT/A – BoNT/G), are potent inhibitors of neurotransmitter release at synapses (Nieman et al., 1994). These toxins act as Zn^{2+}-dependent endoproteases specifically cleaving the SNAREs. TeNT cleaves specifically the vesicle associated membrane protein (VAMP) synaptobrevin. The syntaxins are cleaved by BoNT/C, SNAP-25 is cleaved by BoNT/A, C and E (Hodel, 1998), and VAMPs are cleaved by BoNT/ B, D, F and G (Pellizzari *et al*., 1999). Toxin-mediated SNAREs cleavage prevents the SNARE core complex formation and in the case of synaptobrevin and syntaxin, detaches the SNARE from the membrane (Jahn and Südhof, 1999) However, once the core complex is fully assembled, it is resistant to toxin cleavage (Hayashi *et al*., 1994).

8. Rab and synaptotagmin bind to SNARE proteins

Functional membrane transport implicates not only SNAREs. There are several proteins interacting with SNAREs such as nSec1 which binds to syntaxins or proteins

which bind to the whole SNARE core complex such as NSF and SNAPs which are necessary for the dissociation of the complex.

Rab proteins and in yeast Ypt proteins are GTP-binding proteins involved in membrane fusion. In the absence of the Rab proteins, fusion is blocked. Rab proteins are attached to membranes via two C-terminal geranylgeranyl groups. In mammals more then 40 different Rabs were isolated and localized to specific membranes, a fact which makes them good candidates to play a role in vesicle trafficking specificity. Rabs act through effectors, proteins which bind only to Rab proteins. Rabs and their effectors may link and unlink vesicles from the cytoskeleton (Kato et al., 1996), but the exact role of Rab proteins is still unknown (Jahn, 1999; Lin and Scheller, 2000).

In neuronal cells, calcium triggers exocytosis extremely rapidly i.e. within 200μs. This phenomenon has been recognized already decades ago, but the calcium sensors involved in neurotransmitter release is still unknown. Synaptotagmin I a protein purified from synaptic vesicles, could play a role in synaptic transmission. Knock-out mutants of synaptotagmin produced in *C. elegans*, *Drosophila* and mice showed a decreased amount of calcium-dependent secretion. Synaptotagmin possesses two potential Ca^{2+}-binding domains. Synaptotagmin binds to syntaxin 1A (Chapman *et al*., 1995), SNAP-25 (Schiavo *et al*., 1997) and phospholipids (Li *et al*., 1995) in a calcium-dependent fashion. This suggest that a Ca^{2+} influx causes a change in the binding of synaptotagmin to the SNAREs allowing core complex formation and membrane fusion (Lin and Scheller, 2000).

9. The secretory pathway in yeast

The yeast *Saccharomyces cerevisiae* is an unicellular organism and belongs to the ascomycetes family of fungi. *S. cerevisiae* is an ideal model organism for eukaryotic cell biology because of its small genome size of 12 x 10^6 bp and the short reproduction time of around 90 minutes. The genome of *S. cerevisiae* has been sequenced and 21 SNAREs involved in the endomembrane system have been identified and characterized (Table 2).

The endomembrane system of a yeast cell consists of seven major compartments; the ER, the Golgi complex, the TGN, the prevacuolar compartment, the vacuole, the

TABLE 2 SNARE proteins in yeast adapted from (Pelham, 1999 and Jahn, 1999)

Protein	Type	Predominant localization	Genbank accession Nr	References
Pep12p	Q	Golgi, vacuole, lysosome	Z74944	Becherer *et al.*, 1996,
Vam3p	Q	Vacuole	Q12241	Darsow *et al.*, 1997, Wada *et al.*, 1997
Sso1p	Q	Plasma membrane	Z73588	Aalto *et al.*, 1993
Sso2p	Q	Plasma membrane	Z49808	Aalto *et al.*, 1993
Tlg1p	Q	Late Golgi, endosome	Z71256	Holthuis *et al.*, 1998
Tlg2p	Q	Late Golgi, endosome	Z74760	Holthuis *et al.*, 1998, Abeliovich *et al.*, 1998
Sed5p	Q	ER, *cis*-Golgi	Z73198	Hardwick *et al.*, 1992, Banfield *et al.*, 1994
Ufe1p	Q	ER, *cis*-Golgi	Z74983	Lewis *et al.*, 1996
Sec9p	2Q	Plasma membrane	Z72794	Novick *et al.*, 1993
Spo20p	2Q	Plasma membrane	Z49211	Neiman *et al.*, 1998
Gos1p	Q	ER, Golgi	U11583	McNew *et al.*, 1997
Bos1p	Q	ER, Golgi	Z73250	Shim *et al.*, 1991, Newman *et al.*, 1990
Bet1p	Q ?	ER, Golgi	Z47047	Newman *et al.*, 1990
Vti1p	Q	ER, Golgi, vacuole	Z47815	Holthuis *et al.*, 1998, von Mollard *et al.*, 1990, Lupashin *et al.*, 1997
Vam7p	Q	Vacuole	Z72734	Wada *et al.*, 1992, Ungermann *et al.*, 1998
Sec22p	R	ER, Golgi	U17244	Dascher *et al.*, 1991, Ossig *et al.*, 1991
Nyv1p	R	Vacuole	Z73265	Nichols *et al.*, 1997
Snc1p	R	Plasma membrane	U12980	Gerst *et al.*, 1992, Protopopov *et al.*, 1993
Snc2p	R	Plasma membrane	Z75235	
Ykt6p	R	ER, Golgi	Z28196	McNew *et al.*, 1997, Sogaard *et al.*, 1994
Sft1p	?	?		McNew *et al.*, 1997

endosome (an organelle in which the secretory and endocytic pathways converge) and the plasma membrane. Endosomes are the intersection of endo- and exocytosis which makes the exact identification very difficult. SNAREs are distributed in all these compartments.

There are 8 syntaxins in *S. cerevisiae.* Ufe1p is a syntaxin located at the ER and involved in the retro- and anterograde traffic from Golgi to ER. Ufe1p is an ortholog of the mammalian syntaxins syntaxin 8 and 11. Ufe1p can be found in complexes containing Sec20p, a transmembrane proteins of the ER (Lewis and Pelham, 1996) and Sec22p a v-SNARE (Lewis *et al*., 1997). Sed5p is a syntaxin located to the Golgi complex and is essential for the growth of yeast. Mammalian syntaxin 5 and plant AtSed5 are orthologs of Sed5p. Sed5p associates with a broad range of yeast SNAREs in several distinct complexes. Each of the v-SNAREs Bet1p, Bos1p and Sec22p, involved in ER-to-Golgi transport can form a SNARE complex with Sed5p (Lian and Ferro-Novick, 1993). Sed5p catalyses homotypic fusion between ER-

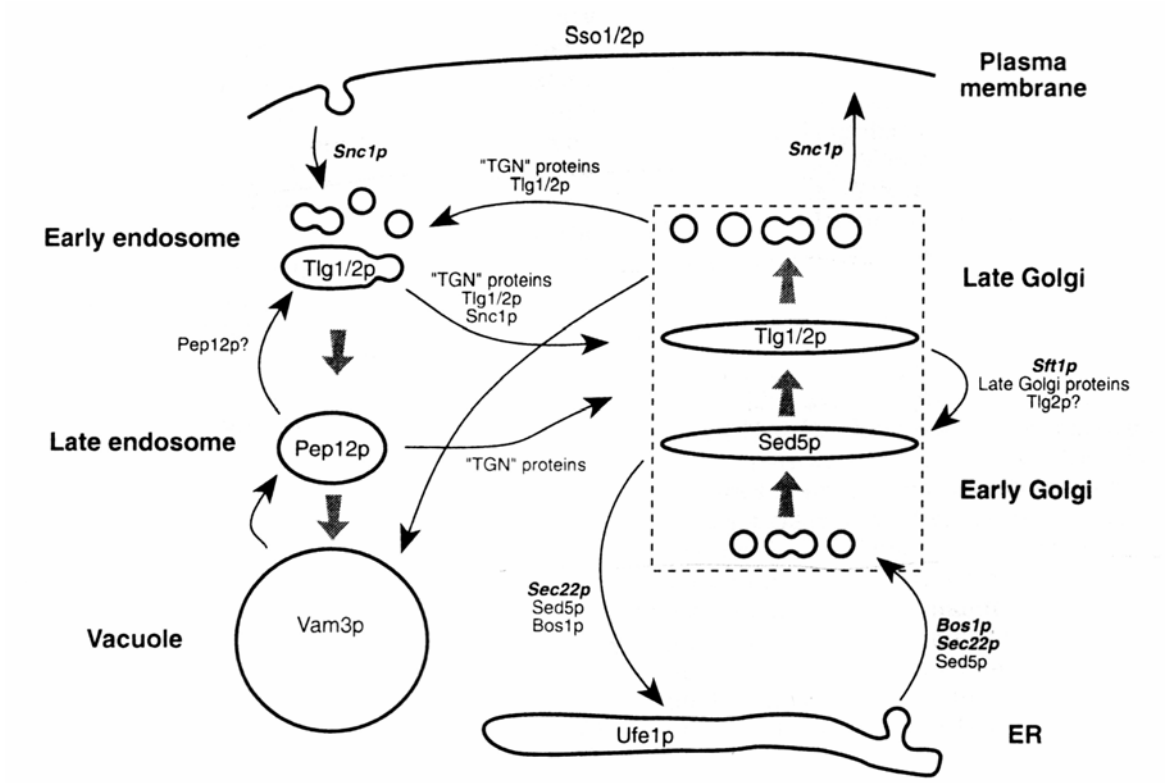

Figure 6: **Schematic outline of the secretory and endocytic pathways of yeast** The syntaxins are showed at the proposed locations. The movements of each syntaxin and the likely vesicular transport pathway are indicated by the thin arrows. The v-SNAREs involved in the corresponding step are indicated in bold italic. The large arrows denote the maturation of a compartment. (Pelham, 1999)

derived vesicles to form an early Golgi cisternae. Sed5p was identified by coimmunoprecipitation with the v-SNAREs Vti1p and Ykt6p as well as with the v-SNAREs Gos1p and Sec22p (Pelham, 1999).

At the late Golgi apparatus or early endosome, there are two other syntaxins Tlg1 and 2p. The exact localization is uncertain because the scattered nature of the Golgi cisternae. In yeast it is hard to distinguish structures from the TGN and the early endosomes. It is likely that Tlg1p and 2p cycle between the Golgi and endocytic compartments. Tlg1 and 2p are involved in the endocytic pathway. Tlg1p, classified as a t-SNARE, coprecipitates with both syntaxins Tlg2p and Sed5p. (Pelham, 1999). Tlg1p interacts with the GTPase Ypt6p which leads to the fusion of endosome-derived vesicles with the late Golgi (Siniossoglou and Pelham, 2001). Tlg2p interacts with the Sec1p-like / Munc-18 protein Vps45p and inhibit spontaneous SNARE complex formation with Tlg1p and Vti1p at the endosomal membrane (Bryant and James, 2001).

The syntaxins Pep12p (ortholog of mammalian syntaxin 7 and plant AtPep12) and Vam3p are located at the late endosomes and vacuoles. Pep12p forms a complex at

the endosomes requiring the v-SNARE Vti1p and the Sec1p-homologue Vps45p. Vam3p interacts with the v-SNARE Vti1p. Vam3p and Vam7p mediate homotypic vacuole fusion. This process requires Sec17p, Sec18p, and the v-SNARE Nyv1p as well as perhaps Vti1p (Sanderfoot and Raikhel, 1999; Figure 6)

The following set of SNAREs functions in exocytosis in yeast. The v-SNARE Snc1 and Snc2p are found in vesicles derived from the late Golgi or the TGN and interact with one of the two redundant syntaxins Sso1p or Sso2p and with the homologue of SNAP-25, Sec9p. Together, these SNAREs form the core complex at the plasma membrane (Grote *et al.*, 2000).

A second SNAP-25 homologue Spo20p was isolated in yeast and is involved in sporulation. Spo20p interacts with the same SNAREs as Sec9p does, to form the SNARE core complex, but is specifically expressed during the budding stage. In contrast, Sec9p is active in the vegetative cell stage (Grote *et al.*, 2000).

In yeast cell which are going to bud another protein complex consisting of the 7 proteins Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo70p was discovered at the pre-bud site, the growing site of the future daughter cell. This protein complex is called the exocyst. The exocyst is localized in subdomains of the plasma membrane that represent sites of active vesicle fusion. An excoyst-like protein complex was also isolated from rat brains indicating that this complex is evolutionarily conserved. The exocyst plays a key role in vesicle targeting (Guo *et al.*, 1999).

Sec18p and Sec17p are homologous to mammalian NSF and α-SNAP respectively. They bind SNARE complexes and dissociate them with the hydrolysis of ATP.

The fact that mammals and plants have supplementary set of SNARE which have no orthologs in yeast, lead to the suggestion that yeast cells possess the ‘basic set’ of SNARE proteins necessary for trafficking through the endomembrane system. The identification of orthologs of SNARE protein in mammalian and plant cells show that the proteins implicated in trafficking along the secretory pathway are probably conserved in all eukaryotic cells (see for review Pelham, 1999 and Sanderfoot and Raikhel, 1999).

10. Secretion in plants

The plant kingdom has developed an impressive diversity over millions of years. The division of flowering plants alone has over 235'000 different species. Out of these, over the last decades *Arabidopsis thaliana* a small unspectacular Brassicacae became one of the most powerful model plant for genetic, developmental and cell biological studies in higher plants. This small, annual plant has a fully sequenced genome with a size of 1.5×10^8 base pairs distributed on 5 chromosomes, a small genomic size compared to other angiosperms. *Arabidopsis* has a short generation time of 2 - 3 months and is self-fertilizing which makes it easy to cultivate. Another advantage of *Arabidopsis* is, that it is easily transformable by *Agrobacterium tumefaciens.*

10.1 Growth and development of plants

The seed consists basically of the embryo, which develops from the fertilized egg, the endosperm where reserves of nutriments are stored and a seed coat. Seeds can be transported by any organism or by wind to a new habitat. Once the condition is favourable the seed starts to germinate. With germination growth of the plantlet starts. There is rupture of the seed coat, the young hypocotyl emerges. The root grows in the soil, and the cotyledons begin to perform photosynthesis. The primary growth originates in the apical meristems of the root and shoot. Meristems are undifferentiated plant tissues, composed of dividing cells from which new tissue arise. With enough light, water, and minerals, the plant will be able to grow until it completes its life cycle. Plant growth is dependent on cell division and cell growth. Cell growth means continuous production of membrane- and cell wall components that is mediated by the secretory pathway.

Secretion is not involved only in cell growth, there are differentiated tissues and organs with a high secretory activity.
In maize, cells of the root cap secrete actively slime or 'mucigel' composed of complex polysaccharides with a high content of fucose and β-1,4-linked glucans. This mucigel is secreted into the extracellular space and protects the roots from damage

and injuries when they are growing into the soil. The increased secretory activity in these cells is reflected in the subcellular structure. For example, some Golgi stacks have distended cisternae, or more vesicles that are fusing with the plasma membrane (Whaley et al., 1959).

Among gland cells, there is a wide variety of secretory cell types like nectaries, hydathodes, salt glands or digestive glands of insectivorous plants. They secrete sugars as in nectaries (Findlay, 1988), polysaccharides or proteins as in carnivorous plants by ER or Golgi mediated vesicles (Robins and Juniper, 1980).

Another highly secreting tissue is the cereal aleurone layer. All cereal grains have this small, thick-walled cell layer around the starchy endosperm. During germination or gibberellic acid treatment, the aleurone layer produces hydrolytic enzymes which are secreted in the endosperm where they degrade starch, proteins and cell walls. The products are then taken up and utilized by the growing plantlet. The most abundant enzyme is α-amylase which represents 30% of total protein synthesis (see review by Battey and Blackbourn, 1993).

Polarized growth in single cells like root hairs which elongate from root epidermis cells or pollen tubes growing from the stigmatic surface to the ovule, is dependent on a high secretory activity. The polarized growth is characterized by tip growth, a process where vesicles fuse at the tip at rates of up to 6000 per minute in the case of pollen tubes (Thiel and Battey, 1998). Subcellular observations revealed an active cytoplasmic streaming behind the tip of pollen tubes and root hairs (Picton and Steer, 1981). Another characteristic typical of tip growing cells is the distribution of organelles. There are high numbers of vesicles at the tip as well as the presence of several Golgi stacks behind the tip, followed by the ER and the rest of the cell's organelles (Battey and Blackbourn, 1993 and Thiel and Battey, 1998).

Cytokinesis is a process also dependent on the endomembrane system. Cytokinesis is the division of the cytoplasm into two daughter cells. In higher plants, immediately after mitosis, the main part of cytokinesis is the formation of a new plasma membrane, which stretches from the interior to the periphery of the cell where it fuses with the parental plasma membrane. The material for the new cell wall is transported by vesicles budding from the form Golgi complexes. These vesicles are guided by microfilaments to the place of the future cell plate, the new middle lamella. The vesicles fuse into long tubes forming a network called the fusion-tube-generated membrane network (FTN) which undergoes several morphological and biochemical

changes to form then a tubulo-vesicular network (TVN) before it matures to the new membrane and cell wall (Assad *et al.*, 1997 and Nishihama and Machida, 2001).
Secreted proteins are synthesized at the ER, transported by vesicles to the Golgi apparatus and transported through the Golgi apparatus. At the TGN, they are packaged into vesicles which fuse with the plasma membrane releasing their content into the extracellular space. Secretion is the default pathway of soluble secreted proteins. The precursors of many components of the cell wall are synthesized in the Golgi and then transported similarly through the secretory pathway to the extracellular space. The fusion of vesicles at different steps of the secretory pathway is mediated by membrane associated SNARE proteins (Buchanan *et al.*, 2000). In plants the identification and characterisation of SNARE proteins is in the process to become as detailed as in the animal and yeast systems.

10.2 t- and v-SNAREs in plants

Sequencing of the genome of *Arabidopsis* uncovered a remarkable number of SNARE proteins but the precise function of most of them is not known. As in animals and yeast, *Arabidopsis* plants have many t- and v-SNAREs. Some are orthologs of known mammalian or yeast SNAREs, others are only found in *Arabidopsis* (Table 3).

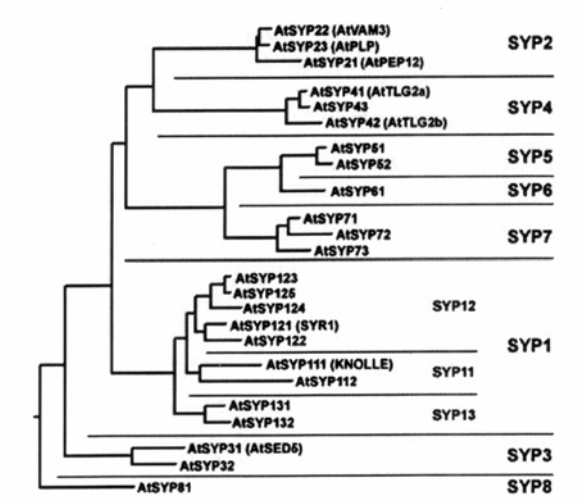

Figure 7 **Arabidopsis syntaxin classes** The protein sequence alignment of all identified *Arabidopsis thaliana* syntaxins revealed a phylogenetic tree with eight classes (SYP1 - SYP8) For those Arabidopsis syntaxins that have been previously published, the prior name is given in parenthesis (Sanderfoot *et al*., 2000).

Twenty-four syntaxins were found by homology and analysis of common structural features. Sanderfoot *et al.*, (2000) classified these syntaxins in eight classes AtSYP1 – AtSYP8, based on their sequences and intron – exon structure (Figure 7). SYP stands for syntaxin of plant. Only 8 plant syntaxins are characterized yet.
The first class of syntaxins AtSYP1 has 9 members and is subdivided in

three gene families based on their splicing pattern AtSYP11 (2 members), AtSYP12 (5 members) and AtSYP13 (2 members) (Sanderfoot *et al.*, 2000). AtSYP111 (KNOLLE) and AtSYP121 (AtSYR1) both homologues to the yeast Sso1/2p, are the only two characterized members in this class. KNOLLE (Lukowitz *et al.*, 1996) is expressed only during cell division and is localized to the phragmoplast (Lauber *et al.*, 1997). The Knolle mutant is lethal at the seedling stage (Lukowitz *et al*., 1996). KNOLLE interacts with the t-SNARE AtSNAP33 which is a homologue of SNAP-25 (Heese *et al.*, 2001).

AtSYP121 (AtSYR1) has an overall identity of 72% with the syntaxin NtSYR1 from tobacco. NtSYR1 is involved in ABA signal transduction in guard cells (Leyman *et al.*, 1999) and is found at the plasma membrane (Leyman *et al.*, 2000). NtSYR1 is interacting with AtSNAP33 as shown by affinity chromatography (Kargul *et al.*, 2001).

In the case of AtSYP112 it is uncertain if the gene is expressed, in contrast to AtSYP122 – 125 for which expressed sequence tags (EST) and cloned cDNAs are present. AtSYP131 / 132 have not been examined (Sanderfoot *et al.*, 2000).

The AtSYP2 class has three members, AtSYP21 (AtPep12), AtSYP22 (AtVAM3), and AtSYP23 (AtPLP), orthologs of the yeast Pep12p and of mammalian syntaxins 7 and 13, respectively. AtSYP21 is functionally complementing the yeast *pep12* mutant. In yeast Pep12p is involved in protein transport to the vacuole and localized to the late endosome. AtSYP21 is expressed in roots and involved in the pathway from the TGN to the PVC and likely localized to a PVC in *Arabidopsis* roots. (Conceição *et al.*, 1997). AtSYP21 forms a 20S complex in *Arabidopsis* that has characteristics of complexes involved in vesicle fusion. Moreover the complex containing AtSYP21 is able to bind to α-SNAP. This is an indication that AtSYP21 can function as a SNAP receptor (Bassham and Raikhel, 1999).

AtSYP22 (AtVAM3) was reported to be localized to the vacuole in the shoot apical meristem (Sato *et al.*, 1997) as well as exclusively at the PVC in vegetative cells (Sanderfoot *et al.*, 1999b). The determination of the definitive localization of AtSYP22 needs further analysis.

The third member of the SYP 2 class is AtSYP23 (AtPLP) which is homologous to AtSYP21 and AtSYP22 and expressed at higher level in leaves, flowers and stems than in roots. The expression pattern of *AtSyp23* transcripts is different from *AtSyp21* indicating another role in vesicle trafficking in plant cells (Zheng *et al.*, 1999).

The class of SYP3 has two members, AtSYP31 and AtSYP32, who are expressed but which function and localization is unknown. AtSYP31 and AtSYP32 are homologous to the yeast Sep5p and to mammalian syntaxin5 both localized to the Golgi apparatus (Sanderfoot *et al.*, 2000).
AtSYP41 (AtTLG2a), AtSYP42 (AtTLG2b) and AtSYP43 are members of the SYP4 class. AtSYP41 and AtSYP42 are localized to distinct domains of the TGN (Bassham et al., 2000) and are orthologs of the yeast Tlg2p and of the mammalian syntaxin 16 (Sanderfoot *et al.*, 2000).
The members of the SYP6 (1 member), SYP7 (3 members) and SYP8 (1 member) classes are found as ESTs or isolated cDNAs but their function and localization is not yet analysed. The SYP7 class has no orthologs in yeast or in mammalian cells indicating that the members of this class are syntaxins specific to plants. AtSYP6 is related to the mammalian syntaxin 6 and 10. Syntaxin 6 is involved in TGN to late endosome trafficking in mammalian cells. AtSYP8 is related to the ER-localized syntaxin Ufe1p from yeast and to the mammalian syntaxin 18 (Sanderfoot *et al.*, 2000).
The different localizations and expression patterns, like AtSYP21 and AtSYP23 which are expressed in different tissues of *Arabidopsis*, of syntaxins are indicating that they posses distinct functions. Gene disruption of individual *Arabidopsis* syntaxins of the AtSYP2 and AtSYP4 groups (AtSYP21, AtSYP22, AtSYP41 and AtSYP42) led to lethality, indicating that each of these syntaxins has a unique and essential function (Sanderfoot et al., 2001).

AtSNAP33, AtSNAP30 and AtSNAP29 are the three members of the second t-SNARE subfamily, the SNAP25-like proteins. There were identified by sequence analysis of the *Arabidopsis* genome. AtSNAP33 behaves as an integral membrane protein and is located at the phragmoplast and at the plasma membrane. It interacts with the syntaxin KNOLLE located to the phragmoplast (Heese *et al.*, 2001) and with NtSYR1, the AtSYP121 homologue from tobacco, localized at the plasma membrane (Kargul *et al.*, 2001). AtSNAP30 and AtSNAP29 are probably expressed but their function and localization is unknown.

A whole set of over 20 expressed v-SNAREs are found in the genome of *Arabidopsis*. They are subdivided in groups corresponding to the yeast orthologs, Vtip, Gos1p, Bet1p, Membrin and VAMP (Sanderfoot et al., 2000; see Table 3).

V-SNAREs in plants have a similarly structure as their homologues in animals and yeast. They are anchored to membranes by a C-terminal transmembrane domain. The SNARE motif, which is necessary for successful SNARE core complex formation is in the center of the protein. AtVTI1a / b are homologues to the yeast v-SNARE Vti1p which is required for multiple transport steps in yeast. AtVTI1a localizes at the TGN and PVC and functions as a v-SNARE responsible for the targeting of vesicles from the TGN to the PVC (Zheng H et al., 1999b).

Another v-SNARE was isolated from suspension-cultured carrot cells. It consists of a 18 kDa polypeptide present in non-clathrin-coated vesicles and proteolyzed by tetanus toxin (Gasparian *et al.*, 2000).

TABLE 3 SNARE proteins in *Arabidopsis thaliana* adapted from (Sanderfoot *et al.*, 2000)

Protein	Type	Predominant localization	Genbank accession Nr	References
AtSYP111 (KNOLLE)	Q	phragmoplast	At1g08350	Lukowitz *et al.*, 1996
AtSYP121 (AtSYR1)	Q	plasma membrane	At3g11820	Leyman *et al.*, 1999
AtSYP122	Q		At3g52400	X. Gansel and L. Sticher
AtSYP21 (AtPEP12)	Q	PVC	At5g16830	Bassham *et al.*, 1995
AtSYP22 (AtVAM3)	Q	PVC / vacuole	At5g46860	Sato *et al.*, 1997
AtSYP23 (AtPLP)	Q		At4g177730	Zheng *et al.*, 1999a
AtSYP41 (AtTLG2a)	Q	TGN	At5g26980	Bassham *et al.*, 2000
AtSYP42 (AtTLG2b)	Q	TGN	At4g33400	Bassham *et al.*, 2000
AtSYP43	Q		At3g05710	X. Gansel and L. Sricher
AtSNAP33	Q	plasma membrane	At5g61210	X. Gansel and L. Sticher, Heese et al., 2001
AtSNAP30	Q		At1g13530	
AtSNAP29	Q		At5g07880	C. Dickey and S. Bednarek
AtVTI11	Q	TGN / PVC	At5g38510	Zheng et al., 1999b
AtVTI12	Q	TGN / PVC	At1g25740	Zheng et al., 1999b
AtVAMP711	R		At4g32150	D.M. Nikoloff and C. Somerville
AtVAMP721	R		At1g04630	M. Schena and R:W: Davis
AtVAMP722	R		At2g33120	D.M. Nikoloff and C. Somerville

10.3 SNARE binding proteins in plants

NSF and SNAPs are two soluble proteins which interact with the SNARE core complex. In the presence of ATP they are able to dissociate the complex, freeing the components. In *Arabidopsis* as well as in all other eukaryotic cells NSF is a single copy gene. Two SNAP proteins are found in *Arabidopsis*, one α-SNAP and one γ-SNAP type according to the classification of mammalian SNAP proteins (Sanderfoot et al., 2000).

The Sec1-family is another group of SNARE binding proteins. *Arabidopsis* contains six members of this family. Among these, only KEULE and AtVPS45 are well characterized. KEULE is involved in cytokinesis and binds to the cytokinesis-specific syntaxin KNOLLE (Assaad *et al.*, 2001). The *keule* mutant is lethal at a seedling stage (Assaad *et al*., 2001). AtVPS45 is able to complement the yeast *vps45* mutant. AtVPS45 is a peripheral membrane protein localized to the TGN. AtVPS45 interacts with the syntaxins AtSYP41 and AtSYP42 (AtTLG2a/b) which are also localized at the TGN (Bassham *et al.*, 2000).

10.4 Regulation of secretion in plants

Animal cells have two different types of secretion. Constitutive secretion is the steady flow of secretory vesicles in the anterograde stream from the ER to the Golgi toward the cell surface. Regulated secretion is the simultaneously fusion of preformed vesicles with the target membrane upon a determined signal. Constitutive secretion takes place in each animal cell whereas regulated secretion is best characterized in neuronal cells. (Battey *et al.*, 1999, Jahn, 1999).
In plant it is thought that secretion is only of the constitutive type and that a regulated secretion as described for neuronal cells does not exist. This belief is based on the observation of the secretion of polysaccharides from root cap cells or the production and secretion of sugars from nectaries. But indeed constitutive secretion is also regulated.

Hormones have an influence on the secretion activity. Auxin stimulates cell growth. The elongation of the cell is accompanied by an increase in H^+-ATPase activity acidifying the cell wall which increases the plasticity of the wall matrix. Cell growth, however, requires increased secretion of membrane material and the release of cell wall components (Blatt *et al.*, 1999). Inhibitors of the secretory pathway like brefeldin A abolish auxin–stimulated cell expansion, indicating that auxin is involved in regulating secretion (Schindler et al., 1994).

Gibberellic acid (GA) induces secretion of hydrolytic enzymes mainly α-amylase in barley aleurone layers. In this tissue the stimulus initiated by gibberellic acid is transduced by an increase in the concentration of intracellular Ca^{2+}, $[Ca^{2+}]_i$. (Bush and Jones, 1988) The increase of $[Ca^{2+}]_i$ provokes secretion. This was shown by patch-clamp measurements of maize coleoptiles after photolysis of the caged Ca^{2+} compound dimethoxynirophenamine. The increase in cytosolic Ca^{2+} induced an increase of the number of vesicles fusing with the plasma membrane (Sutter *et al.*, 2000).

Abscisic acid (ABA) controls the aperture of stomates in higher plants. Stomates are pores on the leaf surface surrounded by two guard cells and are responsible for the gaseous exchange between the leaves and the outer atmosphere. Closure of the pore is triggered by ABA and is driven by the loss of osmotically active solutes and of turgor from the guard cells. During closure of the pore the guard cells show a reduction of 50% or more in the plasma membrane surface. Such effects on membrane surface are more than lateral expansion and compression of the lipid bilayer. It implies a coordinate regulation of exo- and endocytosis during stomatal movement (Blatt, 2000).

Electrophysiological experiments with protoplasts of *Vicia faba* have shown that a hydrostatic pressure provokes a steady increase in plasma membrane capacitance, an indicator for the increase of the cell surface, whereas the specific membrane capacitance of the membrane was not affected. This increase of the cell surface is only explainable by continuous fusion of vesicles with the plasma membrane. The reversible phenomenon was observed as well, applying a negative pression on the cell. Then, the protoplast started to shrink. The shrinking increase the number of endocytic vesicles which are cut off from the plasma membrane. Vesicle fusion and fission induced by the hydrostatic pressure is independent of $[Ca^{2+}]_i$ (Homann, 1998).

The data on GA and hydrostatic pressure suggest that two pathway exist for vesicle trafficking to the plasma membrane, one which is $[Ca^{2+}]_i$ dependent and one which is $[Ca^{2+}]_i$ independent. It is not excluded that in plants different control mechanisms function on different pools of vesicles ready to fuse with the plasma membrane (Blatt *et al.*, 1999, Sutter *et al.*, 2000).

Secretion is a complex process which is regulated at different levels. The transcription and translation of proteins necessary for vesicle fusion or proteins transported by the secretory pathway are also regulated processes in a plant cell and are not discussed here. Moreover, in mammalian cells SNARE proteins can undergo posttranslational modification regulating their activity (Lin and Scheller, 2000). Phosphorylation of syntaxin 1A does not affect the SNARE core complex formation but enhances its interaction with synaptotagmin (Risinger and Bennett, 1999). Phosphorylated SNAP-25 shows a 38% inhibition of the interaction with syntaxin 1A (Shimazaki *et al.*, 1996). These posttranslational modifications together with hormones and other stimuli are responsible for the complex regulation of secretion in eukaryotic cells.

11. The response to mechanical stimulation in *Arabidopsis*

Plants cannot escape from their place, when the environmental conditions change. They do not have a developed neuronal system which transmits changes in their environment as animals have. But plant are also sensitive to environmental stimuli like wind, cold, heat and mechanical stimuli as touch. Plants exposed to wind or touch grow shorter and stockier and become stronger. These developmental changes are called thigmomorphogenesis (Braam *et al*., 1997). The touch genes (TCH) are found by accident, looking for genes increased in *Arabidopsis* sprayed with gibberellins (Braam and Davis, 1990). These genes are not induced only after spraying with water and touch, some of them are expressed in darkness, temperature shocks and hormonal treatment (Jaffe, 1973).

The TCH genes have the same expression pattern; a strong and rapid increase in gene transcripts (within minutes) and gene expression is then rapidly turned off (Braam and Davis, 1990). Two models of regulation are proposed by Braam et al. (1997). One postulates that the perception of different stimuli leads to the activation

of different signal transduction pathways for the upregulation of the TCH genes by different regulatory elements. The second model postulates that the perception of different stimuli leads to the activation of signal pathways that converge to a common regulation factor upstream to the TCH gene expression.

Changes in free cytosolic calcium concentrations is a potential common messenger mediating TCH gene induction. Cultured *Arabidopsis* cells were exposed to an increased external [Ca^{2+}] which causes an induction of the expression of TCH genes (Braam, 1992). Chelating Ca^{2+} by EGTA or BAPTA inhibits the expression of the TCH genes after heat or cold shock (Polisensky and Braam, 1996). The correlation between increases in cytosolic [Ca^{2+}] and the induction of the TCH gene transcripts indicated that the TCH genes are regulated as postulated in the second model described above (Braam *et al*., 1997). These results are not enough to describe the whole signal transduction pathway after mechanical stimulation. The hormone ethylene could be another key player, since exogenous ethylene leads to thigmomorphogenetic-like changes (Johnson *et al*., 1998). To determine whether ethylene has a role in plant responses to mechanical stimulation the ethylene-insensitive *Arabidopsis* mutants *etr1* and *ein2* were exposed to wind. They responded similarly as the wild type with a delay in flowering, decrease in inflorescence elongation rate, shorter mature primary inflorescences and appropriate TCH gene expression changes. ETR1 and EIN2 are not involved in thigmomorphogenesis and TCH gene expression (Johnson *et al.*, 1998).

TCH1 encodes calmodulin. Calmodulins bind 4 Ca^{2+} ions which leads to a change of conformation (Braam *et al*., 1997). In this conformation calmodulin can interact with and modify a variety of target proteins (for review, see Roberts and Harmon, 1992). TCH2 belongs to the calmodulin family and contains four Ca2+ binding sites (Khan *et al*., 1997). TCH3 encodes a calmodulin-related protein which can bind up to 6 Ca^{2+} ions. TCH3 is expressed and localized in cells of the vascular tissue and in cells undergoing expansion (Antosiewicz *et al*., 1995). These tissues are exposed to pressure and tension during morphogenesis. The authors postulate that TCH3 could be involved in cell or tissue reinforcement and / or play a role in vesicular traffic regulating the secretion of structural components of the cell wall (Braam *et al.*, 1997). TCH4 encodes a xyloglycan endotransglycosylase (EXT), a cell wall-modifiying enzyme. The cell wall is composed of cellulose which are embedded in a matrix of hemicellulose, pectins and proteins. The major hemicellulose in dictyledonous

species is xyloglucan which consist of (1-4)-β-linked D-glucosyl residues. EXT cleave xyloglucan polymers internally and ligate the newly generated reducing end to another xyloglucan chain (Fanutti *et al*., 1993). Controlling these modifications EXT may have an influence on the determination of the properties of the cell wall such as extensibility, strength and integrity (Braam *et al.*, 1997; see for review Buchanan *et al*., 2000). TCH4 is member of a multigene family in Arabidopsis, but is the only member which is induced after environmental stimuli like darkness, heat- and cold shock and touch (Braam *et al.*, 1997).

TCH genes are not a unique to *Arabidopsis* some are also described in other plants. In tomato (*Lycopersicon esculentum*) 1-aminocyclopropane-1-carboxylate synthase (ACC) is also rapidly and transiently expressed after touch and wind (Tatsuki and Mori, 1999). Eight calmodulin genes are isolated and characterized from potato (*Solanum tuberosum*) which are expressed after touch (Takezawa et al., 1995). In wheat a lipoxygenase is expressed after mechanical stimulation (Mauch *et al*., 1997). Probably there are more genes not only in *Arabidopsis* which have a similar expression pattern as the TCH genes.

12. Programmed cell death in plants

Cell death is part of the growth and development in many eukaryotic organisms, as well as a component of the response to biotic and abiotic stresses. In these cases the organism initiates and executes the cell death process. This is referred to as programmed cell death (PCD) (Buchanan *et al*., 2000). The most impressive PCD in plants take place every year in autumn when the leaves undergo senescence and turn to different red colour shades.

PCD is a process that lead to death, during PCD the cells are viable until the final act of death. For example senescence of mesophyll cells goes on until almost all of the leaf's resources have been exported to other parts of the plant.

Cell death influences all phases of the plant life cycle from germination to reproductive development.

12.1 PCD in the life cycle of plants

In the seed the plant embryo is surrounded by a endosperm layer. The endosperm functions to store proteins, lipids and carbohydrates. During germination, the embryo has to break through this cell layer. The endosperm undergoes PCD after imbibition which leads to the breakdown of these cells by PCD and allows the expansion of the embryo. During this PCD an increase in nuclease activity and a degradation of nuclear DNA can be observed (Young and Gallie, 2000).

Tissue differentiation, particularly in the development of xylem tracheary elements, would not function without PCD. The differentiation of living procambium cells into dead tracheary elements, major components of vessels, is necessary for efficient water movement through the plant. Before the *Zinnia* procambium cells differentiate to a trachaery element, a secondary thickening of the cell wall occurs, followed by PCD and the breakdown of the cell organelles (Fukuda, 2000).

Senescence is a process where cells and tissues die, while simultaneously nutrients mainly nitrogen and phosphorus are reclaimed. Typically symptoms of senescence in leaves are yellowing, the reduction of the photosynthetic activity and finally the drop off. Senescence can be initiated by hormones, environment, developmental stages which triggers a signal transduction pathway including gene activation and inactivation. A large number of genes expressed during leaf senescence have been isolated from several species. These senescence-associated genes (SAG) encodes for proteolytic enzymes like cysteine proteases and enzymes involved in chlorophyll and nucleic acid degradation. During senescence the cell structure changes drastically and finally the integrity of subcellular membranes and compartmentalization collapses (Buchanan *et al*., 2000). This cascade of gene activation and inactivation is controlled by senescence inhibitors and accelerators. The strongest promoter of senescence is the plant hormone ethylene. Exogenously applied ethylene induces senescence in leaves and flowers and acts also as inducer of fruit ripening. On the molecular level, ethylene increases the transcription of SAGs. Ethylene induces also his own synthesis in a positive feedback loop. Cytokinins are the major antagonists of ethylene. This is based on the observation that the concentration of cytokinins are decreasing in senescing tissues and that exogenous application of the phytohormone on plant tissues delays the appearance of senescence symptoms (Buchanan *et al*., 2000).

Reproductive development is a process which would not function without PCD. After floral induction in the apical meristem, a selective killing of unwanted sexual organ can be observed in dioecious plants. The production and release of pollen grains relies degeneration and death of the anther tissues. The female egg cell in the ovule is the result of PCD of all meiotic derivatives except the egg. These are some examples which underline the importance of the PCD in plant development (Wu and Cheung, 2000).

12.2 PCD in plant as a response to stress

Plants are exposed to different abiotic and biotic stresses during their life cycle. UV irradiation, water stress, air and soil pollution stress the plant as well as herbivores, insects and microorganisms. PCD can be a strategy for the plant to overcome uncomfortable conditions. The roots of maize form aerenchyma in response to oxygen deficiency when the plants are watered too much. The aerenchyma results from PCD of cortical cells which are located between the endodermis and hypodermis, forming spaces that facilitate the circulation of oxygen in the roots (Buchanan *et al*., 2000).

A well characterized PCD induced by a biotic stress factor is the cell death that occurs in response to pathogen attack called hypersensitive reaction (HR). The host cell death is manifested as a rapid collapse of cells around the site of penetration of the pathogen. HR is programmed genetically in the plant cells and involves the transcription of new genes encoding hydrolytic enzymes. The plants are able to recognize and respond to a pathogen with a HR, if the disease resistant gene (R) interacts with the corresponding specific avirulence gene(Avr) carried by the pathogen (see also chapter 13). The goal of the HR is to kill the host tissue around the infection site to overcome the spreading of the pathogen and protect the uninfected parts of the plant (for a review see Shirasu and Schulze-Liefert, 2000).

12.3 Lesion-mimic mutants

Mutants have been identified in plants that spontaneously form localized areas of death tissue resembling those seen in the HR but in the absence of pathogens. These mutants sustain the hypothesis that HR follows an endogenous cell death program without any input from a pathogen. The reason of lesion formation in this mutants can be either the mutation of genes for which the wild type functions are involved in disease resistance or the mutants cell death phenotype results from perturbed metabolism which interferes with the process of PCD. The lesion mimic mutants can be classified by their phenotype. Some lesion mimic mutants exhibit HR markers like the upregulation of the defence-response while other do not (see also chapter 13).

Another characteristic is the extension of the lesion. The initiation class is one class of mutants which develop discrete areas of necrosis that do not spread. The propagation class have lesions which once formed, spread over the plant. This observations lead to the idea that two separate processes are involved in the development of lesions. The initiation of the lesion can be caused by a misregulation of lesion initiation functions, for example signal receptors or early signal transduction can be disturbed. The second process concern the spreading of lesions over the plant.

Lesion mimic mutants can be found naturally, isolated after mutagenesis or generated by constitutive transgene expression. Transgene-induced lesion mimics may be classified into four different groups: pathogen-derived genes, signal transduction-inducing genes, general metabolism-perturbing genes and killer genes.

Pathogen derived transgenes such as avirulence factors or elicitins expressed in plants can directly interact with the corresponding receptor and activate HR in the same manner as pathogens would. Signal transduction inducing genes are transgenes that may activate or effect different components of the signal transduction pathway involved in programmed cell death. The expression of metabolism perturbing transgenes in plants alters the cellular homeostasis and this perturbation can evoke PCD. The killer genes are genes thought to directly cause cell death. (for a review see Mittler and Rizhsky, 2000).

The lesion mimic mutants are a powerful tool to our understanding of PCD and the signals that control it in plants. PCD-inducing transgenes may provide evidences to find entry points / crosstalks into the cell death pathway.

12.4 The role of reactive oxygen intermediates in PCD

The production of reactive oxygen intermediates (ROI) were described after pathogen inoculation. The plant cells produce superoxide by NAD(P)H oxidases and / or peroxidases a few hours after pathogen inoculation. Superoxide itself is not particularly toxic but it can be enzymatically converted in hydrogen peroxide which can cross membranes and can be converted into even more noxious ROI. This oxidative burst is observed in compatible as well in incompatible plant – pathogen interactions. The accumulation of ROIs can directly cause cell death or act as a signal and trigger an independent cell death pathway or transcription of defence genes (Figure 8 for review see Heath, 2000; Shirasu and Schulze-Lefert, 2000).
Antioxidants, molecules which absorb free electrons can inhibit the initiation and propagation of PCD. Diphenyleneiodonium (DPI) is an inhibitor of the plasma membrane NAD(P)H oxidase. In leaves of the *Arabidopsis* mutant *lsd1* (lesions simulating disease) injection of DPI reduces in a dose-dependent manner lesion formation compared to the untreated *lsd1* leaves (Jabs *et al*., 1996). Ascorbate, butylated hydroxy toluene (BHT) or the reductant dithiothreitol (DTT) and KI are described to reduce the rate of light induced cell death in barley aleurone protoplast (Bethke and Jones, 2001).

13. Plant – pathogen interaction

Plants are exposed to continuous attack from bacteria, viruses, fungi, invertebrates and herbivores. Because their immobility precludes escape, plants developed constitutive and inducible barriers against pathogens. The constitutive barriers are the cell wall, a complex matrix based on cellulose, pectin and phenolic compounds and the cuticle a lipophilic layer of the outer extracellular matrix of the epidermis (Sieber et al 2000).
A pathogen is defined as an organism that grows inside the plant to complete a part or all of its life cycle and in doing so has a detrimental effect on the plant. Most of the pathogens cannot affect most of the plants. But some pathogens are a real problem in agriculture. *Phytophtora infestans* the cause of the late blight disease in potato destroys worldwide kilotonnes of potato crop yield per year. In the 19th century, a late

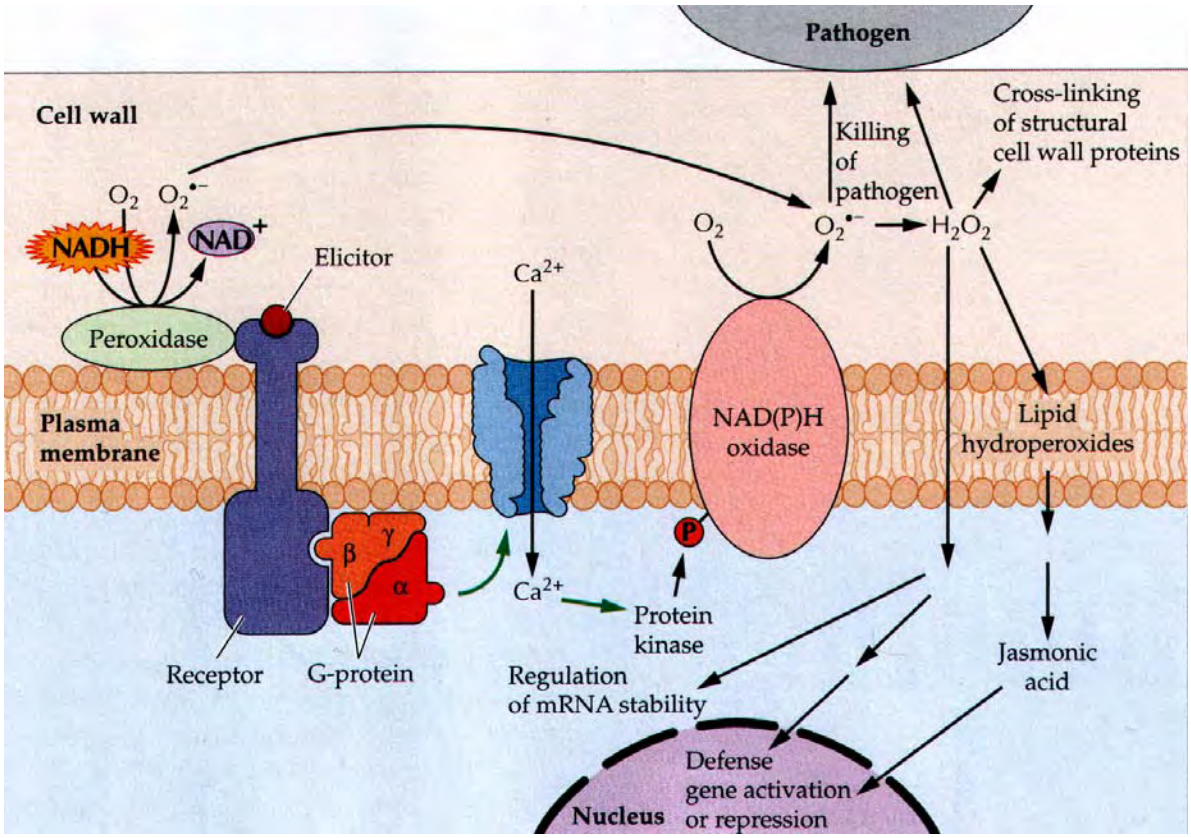

Figure 8 **A possible relationship between pathogen attack on a host cell and the signal transduction pathways** The presence of a pathogen provokes the formation of superoxide by NAD(P)H oxidase and peroxidases. This is called the oxidative burst. The superoxide will be transformed into reactive oxygen intermediates which triggers defence gene expression. (Buchanan *et al*., 2000)

blight disease epidemic destroyed the whole potato yield in Ireland and thousand of people died or were obliged to emigrate. Today only the use of agrochemicals controls the disease but these chemical compounds have also negative effects on the environment.

13.1 Local and systemic plant defence strategies

Potentially all tissues of a plant can be attacked by microorganisms. The pathogens have evolved different ways to invade plants. The most aggressive pathogens are able to penetrate the surface layers of the plant by the use of hydrolytic enzymes or mechanical pressure. Others invade the plant tissue by natural openings like the stomata. A third group can invade only plant tissues that were previously wounded.

The plants evolved different defence systems against pathogens. The plant possesses preformed structural barriers or toxic compounds that confine successful infection to specialized pathogen species. If the pathogen cannot overcome these constitutive barriers then the plant – pathogen interaction is a nonhost resistance.

The plants have not only constitutive barriers they also developed inducible defence systems. The plant can react locally on a pathogen attack by different responses. The lignification of the cell wall of the cell surrounding the infection site prevents the penetration of a fungal hyphae in the cell. Lignin is a phenolic compound which is made from phenylalanine synthesised by the Shikimate-pathway. The precursors are secreted in the cell wall and polymerised to a network by peroxidases.

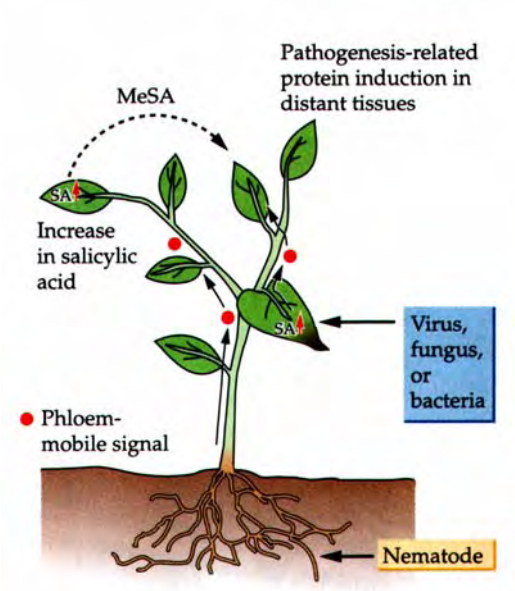

Figure 9 **Systemic acquired resistance** A local necrosis (HR) in the plant initiated by virus, fungus or bacteria triggers a local increase in salicylic acid (SA) accumulation and the formation of a phloem-mobile signal. The signal induces a SA and volatile methyl-SA accumulation in the systemic part of the plant which induce the synthesis of pathogenesis-related proteins in the noninvaded parts of the plant (Buchanan *et al*., 2000).

The production of phytoalexins by the plant after the infection can inhibit the growth of the pathogen and protect the plant against a secondary infection by another pathogen. Phenolics, terpenoids or fatty acid derivates are typical phytoalexins.

Another inducible defence reaction is the expression of pathogenesis related proteins (PRs) by the plant during infection. These genes encode proteins which can inhibit the growth of pathogens. In tobacco, several PR proteins were isolated and described. Some of them encode for hydrolytic enzymes like chitinase (PR-3), β-1,3-glucanase (PR-2), thaumatin like protein (PR-5) for others like PR-1 the function is still unknown (for a review see Bol *et al*., 1990). PR proteins were isolated also in other plants like cucumber (Métraux *et al.*, 1986) or Arabiposis (Uknes *et al*., 1992). Some of these PR proteins like *Arabidopsis* PR-1, PR-2 and PR-5 are secreted in the extracellular space (Uknes *et al*., 1994) where they act on the pathogens before they can penetrate in the plant cell. PR proteins are efficient against a broad range of pathogens, bacteria, viruses and fungi (Uknes *et al*., 1994).

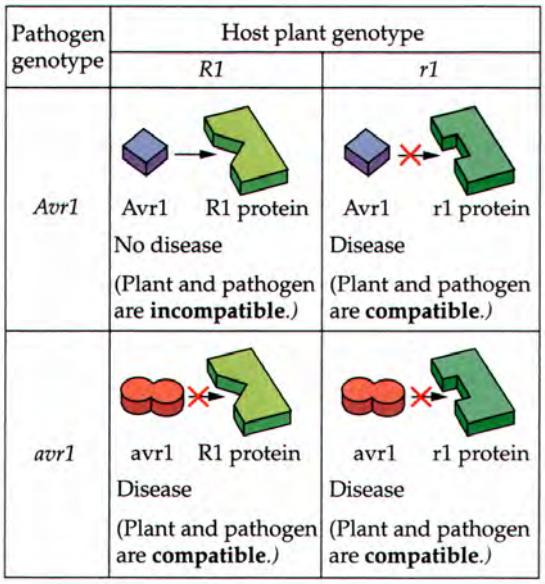

Figure 10 **Flor's gene-for-gene model** Only in the presence of a complementary pairs of a resistance gene (R) and an avirulence gene (Avr) an incompatible plant -pathogen interaction or resistance occur. An alteration or loss of the plant R gene or of the pathogen Avr gene leads to a compatible interaction thus disease (Buchanan *et al*., 2000).

The formation of a hypersensitive reaction (HR) is a well studied defence reaction (for a review see Buchanan *et al*., 2000). HR is a fast collapse of the infected and of some surrounding cells. This programmed cell death prevents the spreading of the pathogen and triggers defences reaction like the formation of PRs in the neighbourhood.

The success of the induced defence mechanisms depends on the race specific interaction between the invading pathogen and the reactions of the plant. In a compatible interaction, the virulent pathogen is not recognized or too late, the plant will be infected and the pathogen is able to complete his life cycle. In the case of avirulent pathogens, plant recognizes the microbe rapidly and induces resistance mechanism which act against the invader and prevents that the pathogen can complete his life cycle.

In 1940 Flor observed that different isolates of flax plants were resistant to some isolates of the flax rust pathogen but not to all. This resistance of the flax plant was inherited as a single dominant trait as well as the virulence of the pathogen. He proposed a gene-for-gene model which predicts that plant resistance will occur only when a plant possesses a resistance gene (R) and the pathogen expresses the complementary avirulence gene (Avr). If one of these genes is absent the plant is not able anymore to recognize the pathogen early enough and the plant becomes susceptible (Figure 10). Jones and collaborators isolated in 1994 in tomato a resistant gene, Cf-9, which confers resistance to infection by races of the fungus *Cladosporium fulvum* that carry the avirulence gene Avr9. Cf-9 encodes a membrane anchored extracytoplasmic glycoprotein which has homology to the leucine-rich repeat family. Further, several R and avr genes were isolated in different organisms (for reviews see Wit, 1995; Buchanan *et al*., 2000).

Plants can induce defence reactions locally as described above and also systematically. This phenomenon is called systemic acquired resistance (SAR) and can be induced by a prior exposure to pathogens, various chemicals or physical stress (Métraux JP *et al*., 1990). Plants inoculated with a pathogen which form an HR produce additionally to the local defence reaction a signal which is translocated to other parts of the plant where it induces defence reactions. A phenolic metabolite, salicylic acid (SA), was shown to be produced by plants locally, at the site of infection, but also found in the phloem sap and in uninfected systemic leaves making SA a possible signal for SAR (Métraux JP *et al*., 1990; Figure 9). SAR is independent of the nature of the initial inoculant and offer a protection against a broad range of pathogens. The pathogen induced SAR is observed in different classes of higher plants. SAR can be induced also by SA treatment or with the functional analogues 2,6-dichloroisoicotinic acid (INA) or benzothiadiazole (BTH; BION) (see for reviews Sticher *et al*., 1997; Métraux, 2001).

A similar phenomenon is induced systemic resistance ISR. Non pathogenic root-colonizing bacteria like rhizobacteria have also been found to induce resistance in leaves (Pieterse *et al*., 1998).

13. 2 Peronospora parasitica, Plectosporium tabacinum and Pseudomanas syringae, are model organisms to study plant – pathogen interactions in *Arabidposis*

The oomycete *Peronospora parasitica* is an obligate biotrophic pathogen. The spores of *P.parasitica* germinate on the leave surfaces and invade the tissue by penetration through the stomata. The hyphae produces haustoria, organs which absorb nutrients from the plant cells. These haustoria grow between the plant cell wall and plasma membrane without disrupting the membrane. After 5 – 7 days the hyphae develop conidiophores through the stomata bearing spores which are distributed further by water and wind. Different isolates of *P. parasitica* are known. The isolate Noco interacts with *Arabidopsis thaliana* ecotype Columbia in a compatible way whereas Emwa interacts in an incompatible way (Koch and Slusarenko, 1990).

Plectosporium tabacinum is a necrotroph, a host cell killer and is the cause of diseases of sunflower, basil, cucurbits as well as peanuts (Palm *et al*., 1995). The spores harvested from cultivated *P. tabacinum* on potato dextrose germinate on the

leaves and penetrate in the tissue and 2- 3 days after inoculation the plant tissue is completely necrotised.

Pseudomonas syringae pv tomato, a bacterial pathogen, was chosen as a model system for molecular genetic analysis of plant-pathogen interactions. *P. syringae* strains were found to be virulent or avirulent on specific *Arabidopsis* ecotypes. *Arabidopsis thaliana* ecotype Columbia is susceptible to *P. syringae* strain DC3000 but resistant to the same strain carrying the avirulence locus avrRpt2. This suggests that a single locus in Columbia determines resistance (Whalen et al., 1991)

All three described pathogen induce an accumulation of SA, the expression of pathogenesis related proteins such as PR-1 or PDF1.2 and camalexins in Arabidopsis (Thomma *et al*., 2001).

13.3 Signal transduction network operating in pathogenesis

Plants are exposed to signals that affect their growth, development and physiology. External signals from the environment such as gravity, light, temperature, wind etc are translated in biochemical signals which allow the plant to react to these stimuli. Signals such as hormones, minerals, peptides, etc can modify the cell metabolism, growth and development.

The first step of transduction of an external or internal signal is the signal perception. The signal can be perceived by a receptor which induces a cascade of reaction such as phosphorylation events leading to the activation of transcription factors which regulate the *de novo* synthesis of proteins. Or a signal molecule can change the membrane potential by modulating an ion channel-linked receptor which can activate a mitogen-activated protein (MAP) kinase cascade. Every stimuli induce a particular signalling cascade which can interfere with other signal transduction processes and sometimes identical secondary messengers are involved for example Ca^{2+}. The field of signalling touches several processes in the plant, but this would be out of the focus of this chapter (for a review see Buchanan *et al*., 2000).

Figure 11 shows a simplified scheme of signal transduction in *Arabidopsis* in response to different pathogens. After initial recognition of the pathogen by the plant, a cascade of reactions that include changes in ions fluxes, phosphorylation events, the production of reactive oxygen intermediates and formation of a signal molecule.

One of these signal molecule is salicylic acid (SA). SA accumulation is observed only in some plant - pathogen interactions. The formation of necrosis due to an avirulent pathogen seems to be a key event to trigger the accumulation of SA and the expression of different defence related proteins such as the pathogenesis related proteins (PRs) associated with resistance to *Pseudomonas syringae, Peronospora parasitica* or *Erysiphe cichoracearum*. Transgenic approaches and mutant screens were a successful tool to reveal components of the SA signal transduction pathway. *Arabidopsis* transformed with the bacterial salicylate hydroxylase (*Nah*G gene) degrades continuously SA. The plants are not able anymore to accumulate SA become susceptible to *P. syringae* (Delaney *et al*., 1994). SID1 (SA induction - deficient) called now EDS5 (enhanced disease susceptibility) is an essential component of the SA dependent signalling pathway and belongs to the MATE-transporter family (Nawrath *et al*., 2002). *Sid1* mutants also do not accumulate SA after pathogen inoculation and become susceptible. *Arabidopsis npr1* mutants (non-expresser of PR genes) are not able to express PRs, even in the presence of SA and are susceptible to pathogen to which wild type were normally resistant (for a review see Métraux, 2001; Sticher *et al*., 1997).

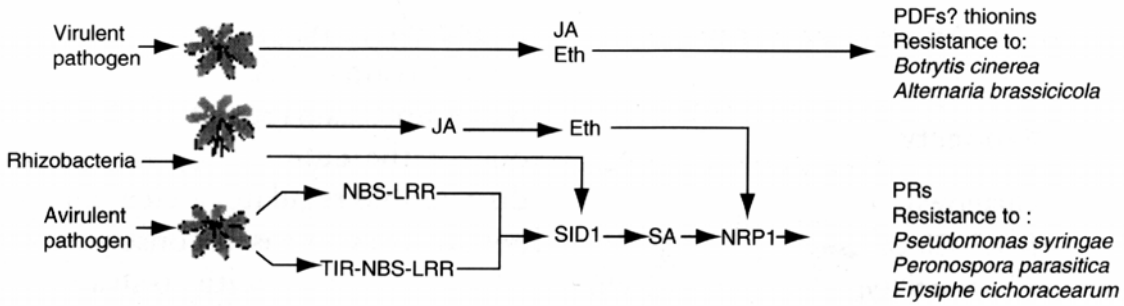

Figure 11 **Simplified diagram of the signal transduction network operating in pathogenesis** The arrows indicate the flow of the signal and proteins from the incoming (left) side and the response (right) side (adapted from Metraux, 2001).
Abbreviations: NBS-LRR: nucleotide-binding site leucine-rich-repeat protein; NPR: non-expresser of PR genes; SID: salicylic acid induction deficient; TIR-NBS-LRR: Toll-interleukin-receptor nucleotide-binding-site leucine-rich-repeat protein; JA: Jasmonic acid; Eth: ethylene; SA: Salicylic acid

SA is not the only signalling compound produced by plants in response to infection. Jasmonic acid (JA) and ethylene (Eth) increased strongly in *Arabidosis* after incoulation of *Alternaria brassicicola*. JA and Eth but not SA lead to the expression of the plant defensin PDF (Penninckx *et al*., 1996). Further studies revealed that SA

and JA / Eth are not linear sequences of events. Recent findings suggest that there are crosstalks or interferences between linear pathway.

14. Aim of the work

The project started by the isolation of the cDNA of the t-SNARE SNAP-25 homologue AtSNAP33 from *Arabidopsis* by Xavier Gansel and Liliane Sticher. With antibodies arised against AtSNAP33 it was first shown that AtSNAP33 was localized at the plasma membrane. Microsomal fractionation confirmed the immunolocalisation and showed that AtSNAP33 behaves as an integral membrane protein (Liliane Sticher unpublished results and Heese *et al*., 2001).

A yeast two hybrid screen with AtSNAP33 as a bait permitted he isolation of several potential partners of AtSNAP33, two novel syntaxins AtSYP122 and AtSYP43 and a motor protein kinesin KatB (Xavier Gansel and Liliane Sticher unpublished results). There were indications that AtSNAP33 was expressed ubiquitously except in leaves where the levels varies between low level and not detectable, but the exact expression pattern was unknown.

This work focuses mainly on the functional characterization and expression of the SNAP-25 homologue AtSNAP33 from *Arabidopsis thaliana.* The second part describes the characterization of the two novel syntaxins AtSYP122 and AtSYP43 previously isolated by a yeast two hybrid screen which potentially interact with AtSNAP33.

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Chapter 1: Functional characterization of the KNOLLE-interacting t-SNARE AtSNAP33 and its role in plant cytokinesis

This chapter is the result of a fruitful collaboration with Maren Heese and Gerd Jürgens from the Zentrum für Molekularbiologie der Pflanzen, University Tübingen, Germany. It summarize parts of the work done by Xavier Gansel and Liliane Sticher on AtSNAP33 and one part of the thesis of Maren Heese.

Xavier Gansel and Liliane Sticher started the project by the isolation of the AtSNAP33 cDNA in *Arabidopsis*. Inmmunolocalisation, cell fractionation of *in vitro* cultured *Arabidopsis* roots and the examination of how AtSNAP33 is associated with the membrane was done by Liliane Sticher.

In parallel Maren Heese identified AtSNAP33 by a yeast two hybrid screen with KNOLLE as a bait. The isolation of a T-DNA insertion in the AtSNAP33 gene by Maren gave a hint of a possible function of AtSNAP33. The immunolocalisation of the tagged AtSNAP33 in root sections correlated with the immunolocalisation performed by Liliane Sticher.

My part to this article was the expression study of AtSNAP33. The promoter of AtSNAP33 was fused with the reporter gene GFP. Arabidopsis plants were transformed and homozygote lines were analysed as shown in Figure 3B of this chapter. The reporter gene analysis allowed us to have a more detailed information about the expression of AtSNAP33 compared to the northern blot analysis which correlating with the results presented in this article.

This collaborations was also the basis for the successful continuation of the functional characterization of AtSNAP33 summarized in the chapter 3.

JCB Article

Functional characterization of the KNOLLE-interacting t-SNARE AtSNAP33 and its role in plant cytokinesis

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Cytokinesis requires membrane fusion during cleavage-furrow ingression in animals and cell plate formation in plants. In *Arabidopsis*, the Sec1 homologue KEULE (KEU) and the cytokinesis-specific syntaxin KNOLLE (KN) cooperate to promote vesicle fusion in the cell division plane. Here, we characterize AtSNAP33, an *Arabidopsis* homologue of the t-SNARE SNAP25, that was identified as a KN interactor in a yeast two-hybrid screen. AtSNAP33 is a ubiquitously expressed membrane-associated protein that accumulated at the plasma membrane and during cell division colocalized with KN at the forming cell plate. A T-DNA insertion in the *AtSNAP33* gene caused loss of AtSNAP33 function, resulting in a lethal dwarf phenotype. *atsnap33* plantlets gradually developed large necrotic lesions on cotyledons and rosette leaves, resembling pathogen-induced cellular responses, and eventually died before flowering. In addition, mutant seedlings displayed cytokinetic defects, and *atsnap33* in combination with the cytokinesis mutant *keu* was embryo lethal. Analysis of the *Arabidopsis* genome revealed two further SNAP25-like proteins that also interacted with KN in the yeast two-hybrid assay. Our results suggest that AtSNAP33, the first SNAP25 homologue characterized in plants, is involved in diverse membrane fusion processes, including cell plate formation, and that AtSNAP33 function in cytokinesis may be replaced partially by other SNAP25 homologues.

Introduction

Cytokinesis completes the process of cell division by forming new membranes that partition the cytoplasm among the daughter cells. Although other aspects of cell division are highly conserved, cytokinesis appears to be carried out very differently between animals and plants (Glotzer, 1997). In animals, a contractile actomyosin-based ring marks the margin of the ingrowing plasma membrane, which closes like a diaphragm in between the newly formed nuclei (Straight and Field, 2000). The ingression of the cleavage furrow is supported by vesicle fusion that inserts new membrane material behind the leading edge (Lecuit and Wieschaus, 2000). By contrast, somatic cytokinesis of higher plants starts in the center of a dividing cell with the de novo formation of a disk-shaped membrane compartment, the cell plate, that grows centrifugally, eventually fusing with the parental plasma membrane (Otegui and Staehelin, 2000). Formation and lateral expansion of the cell plate are brought about by the fusion of Golgi-derived vesicles, initially with one another and later with the margin of the growing cell plate. Thus, although cytokinesis appears to be different between animal and plant cells membrane fusion is required in both (O'Halloran, 2000). However, not much is known in either system about the molecules driving this process.

The machinery of membrane fusion shows a high degree of conservation across eukaryotes and has been studied in a variety of systems (for reviews see Blatt et al., 1999; Pelham, 1999; Lin and Scheller, 2000). Recent models propose that the initial contact between two membrane compartments is made by tethering protein complexes that differ in composition, depending on the membranes involved (Waters and Hughson, 2000). Subsequently, membrane docking occurs, mediated by the formation of a four-helical bundle of membrane-associated soluble *N*-ethylmaleimide–sensitive factor attachment

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protein receptor (SNARE)* proteins. One helix of this trans-SNARE complex is contributed by a vesicle SNARE (v-SNARE) anchored to one membrane compartment, and three helices are added by target membrane SNAREs (t-SNAREs) residing on the other compartment. The t-SNAREs always include a syntaxin, which contributes one helix, whereas the remaining two helices are either from a single SNAP25-type protein or from two separate t-SNARE light chains. Trimeric SNARE complexes involving a SNAP25 homologue have been described in plasma membrane fusion events, whereas tetrameric SNARE complexes prevail in endomembrane fusion processes (Fukuda et al., 2000). To what degree the large class of SNARE proteins contributes to the specificity of membrane fusion processes is still an open question. It has been proposed that possible fusion events are limited by SNARE compatibility and are further restricted in vivo by additional proteins (McNew et al., 2000).

Although SNAREs are sufficient for membrane docking and fusion in vitro (Weber et al., 1998), several regulatory proteins are necessary for trans-SNARE complex formation in vivo. One class of regulators are the Sec1-type proteins. As revealed by x-ray crystallography, neuronal Sec1 binds Syntaxin 1A in a "closed" conformation unsuitable for interaction with other SNAREs and thus may prevent inappropriate SNARE complex formation (Misura et al., 2000). However, yeast Sec1p has been shown to associate with the assembled SNARE core complex (Carr et al., 1999), which suggests several functions for Sec1 or different roles in different systems.

Animal and plant mutants defective in the execution of cytokinesis have been related to genes encoding components of the vesicle fusion machinery. Cellularization of the *Drosophila* embryo is impaired in *syntaxin1* mutants (Burgess et al., 1997), and the cleavage furrow fails to ingress properly in *syntaxin-4* knockout embryos of *Caenorhabditis elegans* (Jantsch-Plunger and Glotzer, 1999). Both syntaxins have been localized to the ingrowing plasma membrane, implying a direct role in membrane addition during cytokinesis. In *Arabidopsis*, mutations in the *KNOLLE (KN)* or *KEULE (KEU)* genes cause strong cytokinetic defects, such as enlarged partially divided multinucleate cells, that are already apparent during embryogenesis and result in seedling lethality (Assaad et al., 1996; Lukowitz et al., 1996). In both mutants, unfused vesicles accumulate in the plane of cell division (Lauber et al., 1997; Waizenegger et al., 2000), suggesting a defect in cytokinetic vesicle fusion. KN belongs to the syntaxin family of proteins and has been shown to localize to the forming cell plate (Lauber et al., 1997). Whereas *KN* expression is confined to dividing cells, *KEU*, which codes for a Sec1 homologue, is expressed more broadly and appears to be involved also in processes other than cytokinesis such as root hair elongation (Assaad et al., 2001). Although several other plant genes with cytokinesis-defective mutant phenotypes have been cloned (for review see Nacry et al., 2000), none encodes an additional component of the membrane fusion machinery. Thus, there are still fundamental questions to be answered. Is the presumed cytokinetic SNARE complex trimeric, tetrameric, or completely different in composition? Are SNAREs other than KN also cytokinesis specific? To identify more components involved in cytokinetic vesicle fusion, we searched for KN-interacting proteins using the yeast two-hybrid system. Here, we report the functional characterization of the KN interactor AtSNAP33 (SNP33), an *Arabidopsis* SNAP25-type t-SNARE of 33 kD.

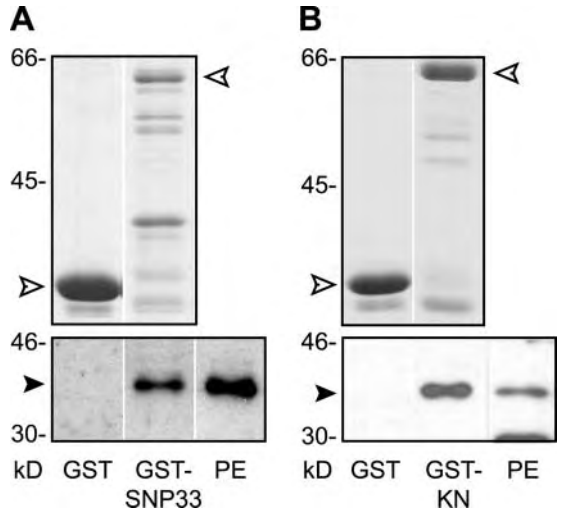

Figure 1. **Binding of SNP33 and KN in vitro.** GST fusion proteins were used to precipitate interacting partners from suspension culture extracts of *Arabidopsis*. GST alone served as a negative control. One third of each precipitate was analyzed by Coomassie-stained gels (top). Note that the amount of GST was the same or more than the amount of GST fusion protein in both assays (white arrowheads). Western blots were used to test for coprecipitated proteins (bottom). PE is an aliquot of the suspension culture extract used for the assay. Black arrowheads indicate sizes of KN (A) and SNP33 (B). (A) Pull-down with GST-SNP33 detected by anti-KN serum; 1/10 of the precipitates and 1/200 of the plant extract (PE) were loaded. (B) Pull-down with GST-KN detected by anti-SNP33 serum; 1/8 of the precipitates and 1/300 of the plant extract were loaded.

Results

AtSNAP33 interacts with the cytokinesis-specific syntaxin KN

SNARE-mediated membrane fusion plays an important role in plant cytokinesis as indicated by the characterization of the Sec1 homologue KEU and the cytokinesis-specific syntaxin KN from *Arabidopsis*. To identify additional members of the presumed SNARE complex involved in cytokinetic vesicle fusion, we performed a yeast two-hybrid screen using the cytoplasmic domain of KN as the "bait" and a "prey" cDNA library prepared from young siliques. Among 1.8 million primary transformants, three independent partial clones derived from the same gene, the *SNAP25* homologue *AtSNAP33* (*SNP33*), were identified as strong KN interactors.

*Abbreviations used in this paper: BFA, brefeldin A; GFP, green fluorescent protein; GST, glutathione *S*-transferase; KEU, KEULE; KN, KNOLLE; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; SNP33, AtSNAP33; t-SNARE, target membrane SNARE; v-SNARE, vesicle SNARE.

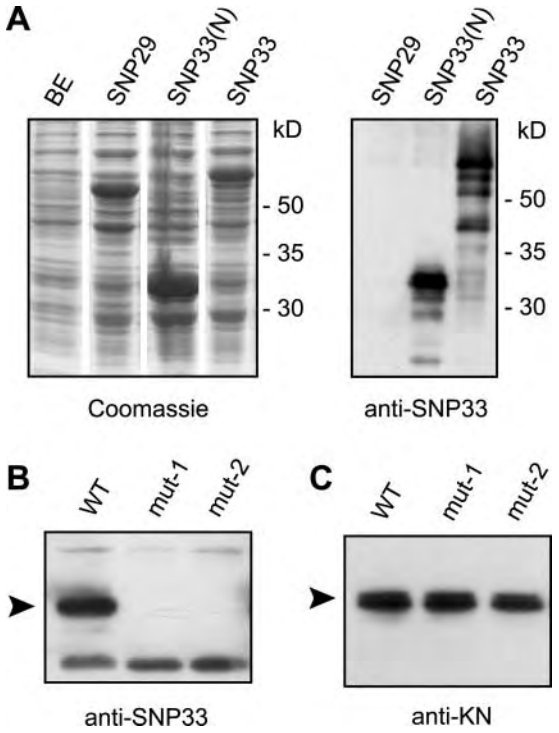

Figure 2. **Characterization of an antiserum raised against SNP33.** (A) The anti-SNP33 serum was tested on extracts from bacteria expressing GST-SNP33 (SNP33), an NH_2-terminal fragment of SNP33 fused to GST (SNP33(N)) or a GST fusion of the related AtSNAP29 (SNP29). The left panel shows a Coomassie-stained gel to compare the amounts of total protein loaded. BE, bacterial extract without recombinant protein. For the Western blot (right panel), 1:200 dilutions of the extracts were used. (B and C) Total protein extracts from wild-type (WT) and two *snp33* (mut-1 and mut-2) mutant callus cultures were separated on SDS-PAGE gels, transferred to PVDF membranes, and detected with anti-SNP33 serum (B) or anti-KN serum (C, control). The arrowheads mark the sizes of the expected proteins. KN expression was the same in all extracts, indicating equal loading. A band of about 33 kD was detected by the anti-SNP33 serum in wild-type but not in *snp33* mutant extracts.

The *SNP33* full-length cDNA encodes a 300 amino acid hydrophilic protein with a deduced molecular weight of 33.6 kD. The COOH-terminal 200 amino acids of SNP33 show 28% identity and 50% similarity to human SNAP25 isoform B, and the regions of highest homology comprise the two α-helical domains required for SNARE core complex formation (see Fig. 9). To rule out artifactual interaction due to protein truncation, full-length *SNP33* cDNA was cloned into the prey vector and analyzed in the yeast two-hybrid assay. Consistently, SNP33 interacted only with KN but not with other unrelated baits (unpublished data).

Glutathione *S*-transferase GST pulldown experiments were performed to assess KN/SNP33 interaction in a different assay. An NH_2-terminal GST fusion of SNP33 bound to glutathione-coupled beads precipitated KN from an *Arabidopsis* suspension culture extract, whereas GST alone did not (Fig. 1 A). A polyclonal antiserum generated against full-length SNP33 (see below) enabled us to perform also the reciprocal experiment. SNP33 was detected in the pellet after precipitation with GST-KN but not with GST alone (Fig. 1 B). In addition, GST-SNP33 specifically precipitated bacterially ex-

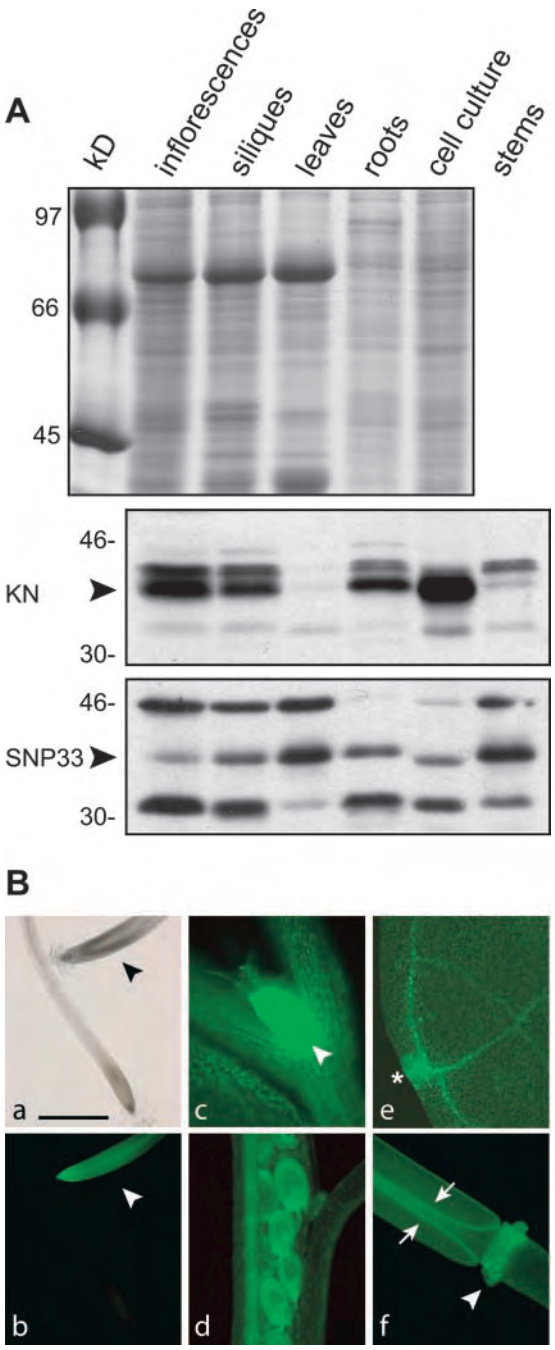

Figure 3. **Ubiquitous expression of SNP33.** (A) Total protein extracts of different organs were separated by SDS-PAGE, transferred to PVDF membranes, and detected with anti-KN serum (second panel; arrowhead, size of KN) or anti-SNP33 serum (third panel; arrowhead, size of SNP33). A Coomassie-stained gel (top) is shown as loading control; protein concentration of stem extract was adjusted to that of cell culture extract based on another Coomassie-stained gel (unpublished data). (B) Expression of GFP under the control of a 2.1-kb genomic fragment containing the *SNP33* promotor (Fig. 4, fragment C) was analyzed. Examples of tissues with strong GFP fluorescence are shown. (a and b) Transgenic root (arrowhead) next to a wild-type root shown in bright field (a) and fluorescence using a GFP filter (b). (c) Young leaves emerging between the petioles of the cotyledons (arrowhead). (d) Ovules in an opened silique. (e) Vascular tissue and hydathode (asterisk) of a cotyledon. (f) Abscission (arrowhead) and dehiscence zones (arrows) of a silique. Bar: (a–d) 150 μm; (e) 75 μm; (f) 950 μm.

pressed $(His)_6$-KN, implying that no other plant proteins are needed for KN/SNP33 interaction (unpublished data).

SNP33 is ubiquitously expressed

For interaction to occur in vivo, SNP33 and KN need to be expressed in overlapping domains. To test for SNP33 expression, we generated a polyclonal antiserum against the full-length protein. The antiserum recognized different forms of recombinant SNP33 protein but not the related AtSNAP29 (Fig. 2 A). On Western blots of plant extracts, a band corresponding to the predicted size of SNP33 was detected. This band was only present in wild-type extracts but not in extracts from a *snp33* T-DNA insertion mutant (see below), confirming the specificity of the anti-SNP33 antiserum (Fig. 2 B).

To compare SNP33 and KN expression, protein blots of extracts from different organs were probed with both antisera (Fig. 3 A). KN expression was restricted to organs containing proliferating tissues as reported previously (Lauber et al., 1997). By contrast, SNP33 seemed to be expressed in all organs analyzed. The expression level in leaves was variable, ranging from barely detectable to high. The reason for this variability of expression, which was observed repeatedly, is not known.

To better resolve the pattern of *SNP33* gene expression, *green fluorescent protein (GFP)* was fused transcriptionally to a 2.1-kb fragment of the *SNP33* promoter region (Fig. 4, construct C). Several independent transgenic *Arabidopsis* lines were tested for GFP expression by fluorescence microscopy, which revealed low level expression in all tissues analyzed (unpublished data). Especially strong fluorescence was observed in root tips, ovules, very young leaves, vascular tissue, hydathodes, stipules, and the abscission and dehiscence zones of the siliques. Examples of tissues with strong expression are shown in Fig. 3 B. In summary, ubiquitous expression of SNP33 allows for in vivo interaction with KN but also suggests other functions unrelated to cytokinesis.

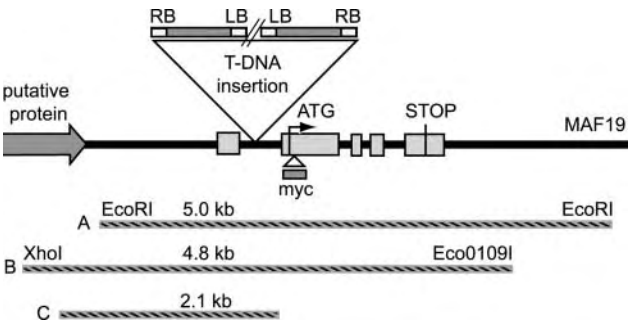

Figure 4. **Schematic representation of the *SNP33* genomic region.** (Top) The black bar represents the genomic region of *SNP33* on chromosome 5 (P1 clone MAF19). The five exons of *SNP33* are depicted as light grey boxes, and the initiating ATG and the stop codon are indicated. The arrow on the left represents a neighboring gene. Triangles indicate the sites of T-DNA and myc-tag insertions (not drawn to scale). (Bottom) The hatched bars represent genomic fragments used for different constructs (lengths and terminal restriction sites are indicated). A and B were used to rescue the *snp33* mutant, and C was transcriptionally fused to GFP for expression analysis of *SNP33*. Note that fragment C comprises a larger 5′ region than rescue construct A and thus likely contains the complete *SNP33* promoter.

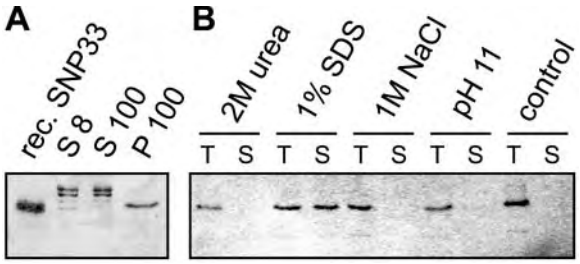

Figure 5. **SNP33 is associated with membranes.** (A) Cell fractionation of in vitro–cultured *Arabidopsis* roots. S8, supernatant of 8,000 *g* precentrifugation; S 100 and P 100, supernatant and pellet of 100,000 *g* ultracentrifugation; rec.SNP33, recombinant SNP33 protein used as a size standard. (B) Aliquots of the microsomal fraction (A, P 100) were extracted with the buffers indicated (0.1 M Na_2CO_3, pH 11; control, 10 mM phosphate buffer, pH 7.6). For each aliquot, total protein (T) before and supernatant (S) after a 100,000 *g* centrifugation were analyzed in Western blots to test for solubilization of SNP33.

Subcellular localization of SNP33 protein

To characterize more closely the in vivo function of SNP33, we analyzed its subcellular localization by cell fractionation and immunofluorescence microscopy. As shown in Fig. 5 A, SNP33 was detected in the pellet after a 100,000 *g* centrifugation step, indicating its association with membranes. Comparable results had been reported for other SNAP25 homologues, although proteins of this family lack a membrane-spanning domain (Brennwald et al., 1994; Steegmaier et al., 1998). To examine how tightly SNP33 was associated with membranes, the pellet of the 100,000 *g* centrifugation was resuspended in different buffers and recentrifuged (Fig. 5 B). SNP33 was released only by detergent but not by high salt, high pH, or urea and thus behaved like an integral membrane protein.

The anti-SNP33 serum was not suitable for immunolocalization of SNP33 protein. Therefore, we generated transgenic *Arabidopsis* plants expressing a myc-tagged version of SNP33. A nucleotide linker coding for the myc epitope was inserted into a unique SalI site of a 4.8-kb genomic *SNP33* fragment (Fig. 4, fragment B), which placed the epitope at amino acid 17 of the SNP33 protein. This construct rescued the *snp33* mutant (see below), suggesting that myc-SNP33 was fully functional.

All plants used for immunofluorescence experiments carried myc-SNP33 as the only functional version of the protein. Transgene expression was analyzed in whole-mount preparations of root tips using the monoclonal c-myc antibody 9E10 (Fig. 6). All cell layers of the root tip were labeled, confirming the ubiquitous SNP33 expression seen in *SNP33–GFP* reporter lines. Whereas no signal was detected in wild-type, *myc-SNP33* plants displayed clear labeling of the plasma membrane, which in most cases was accompanied by a weaker granular staining of the cytoplasm (Fig. 6, A–C).

Dividing cells were analyzed to investigate the role of AtSNAP33 in cytokinesis. Myc-SNP33 was found in a narrow band between the reforming daughter nuclei (Fig. 6 D). Furthermore, SNP33 colocalized with the KN syntaxin at the forming cell plate from early to late in division (Fig. 6, E–G). This observation strongly suggests an in vivo interac-

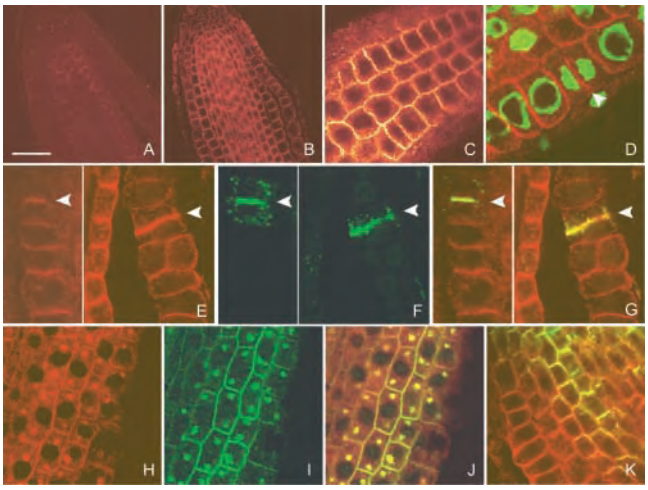

Figure 6. **Immunolocalization of SNP33 protein.** Root tips of wild-type control (A) and transgenic lines expressing myc-SNP33 (B–K) as the only functional version of the protein were analyzed by whole-mount immunofluorescence confocal microscopy. (A–D) Staining for myc-SNP33 (orange/red) and DNA (green, only in D). (A) No expression in wild-type. (B) Expression in all cells of the transgenic root tip. (C) Strong label at the plasma membrane. (D) Labeling of the cell plate (arrowhead) between daughter nuclei. (E–G) Dividing cells were double labeled for myc-SNP33 (E, red) and KN (F, green); overlays (G). Yellow signals in G indicate colocalization of myc-SNP33 and KN. The arrowheads mark early (left) and late (right) cell plates. (H–K) Double labeling for myc-SNP33 (H, red) and PIN1 (I, green); overlays (J and K). Yellow signals indicate colocalization. (H–J) Cells were treated with 100 μM BFA for 2 h. Note the accumulation of label in two distinct patches within the cells. (K) Untreated control cells. Bar: (A and B) 40 μm; (C and K) 15 μm; (D and H–J) 8 μm; (E–G) 10 μm.

tion of both proteins. Analysis of embryonic tissue revealed a subcellular distribution of SNP33 indistinguishable from that seen in the root tip (unpublished data), indicating that the localization of SNP33 is not tissue or developmental stage dependent.

Root tips were treated with brefeldin A (BFA) which blocks anterograde membrane trafficking and leads to an agglomeration of endomembranes (Satiat-Jeunemaitre and Hawes, 1992). Upon treatment, myc-SNP33 accumulated in large patches inside the cell, indicating that at least some of the cytoplasmic label is membrane bound. A similar distribution has been reported for the putative auxin-efflux carrier PIN1 in BFA-treated cells (Steinmann et al., 1999), and the resulting BFA compartment has been interpreted as a mixture of endosome and Golgi stacks (Satiat-Jeunemaitre and Hawes, 1992; Geldner et al., 2001). Colabeling revealed that both PIN1 and SNP33 accumulated in the same compartment (Fig. 6, H–K). In summary, SNP33 is a tightly membrane-associated protein that localizes to the plasma membrane and some endomembrane compartment and to the cell plate in dividing cells.

Identification of an *snp33* T-DNA insertion mutant

The SNP33/KN in vitro interaction and their in vivo colocalization strongly suggested a functional role for SNP33 at the cell plate. To test whether SNP33 function is indeed required for cytokinesis, we searched for mutants in the Versailles collection of *Arabidopsis* T-DNA insertion lines (Bechtold et al., 1993). We identified one line carrying an insertion in the first intron of *SNP33* that separates the transcriptional from the translational start site (Fig. 4). PCR and Southern blot analysis revealed that the insertion most likely consists of two T-DNAs in an inverted repeat conformation. The junctions of both T-DNA right borders with the intron sequences of *SNP33* were confirmed by sequencing (unpublished data).

A recessive mutant phenotype cosegregated with the T-DNA insertion (see below). To determine whether the mutant phenotype was caused by disruption of the *SNP33* gene, plants hemizygous for the T-DNA insertion were transformed with different constructs that contained the *SNP33* genomic region (Fig. 4) and a hygromycin-selectable marker. None of the >100 hygromycin-resistant T1 plants analyzed per construct displayed the mutant phenotype, although ~25% of these plants were homozygous for the T-DNA insertion in the endogenous *SNP33* gene. Furthermore, T1 plants that were heterozygous for the T-DNA insertion at the *SNP33* locus and carried one unlinked rescue construct segregated ~6.25% mutant seedlings, whereas T1 plants homozygous for the T-DNA insertion and carrying one unlinked rescue construct segregated ~25% mutant seedlings. Thus, the observed mutant phenotype was caused by T-DNA disruption of the *SNP33* gene. The overlap of the two smallest rescue constructs defines a 4.1-kb EcoRI/Eco0109I fragment to be sufficient for complementation, containing a 1.2 kb promoter and a 2.2 kb transcribed and a 0.7 kb 3′ region of the *SNP33* gene.

Phenotypic characterization of the *snp33* mutant

Macroscopically, *snp33* mutant seedlings could not be distinguished from wild-type until 7–9 d after germination (Fig. 7 A, left). At that time, brownish lesions appeared on the cotyledons, and the seedlings lagged behind in development. The lesions became more frequent and enlarged until the whole cotyledon turned necrotic. Subsequently, developing rosette leaves gradually formed lesions also, mostly starting at the leaf tip (Fig. 7 B). Although *snp33* mutants grown on agar plates continued to produce rosette leaves for several weeks, they hardly increased in size, resembling extreme dwarfs (Fig. 7 C). Eventually, cell death occurred also in the youngest leaves and in the hypocotyl, and the plantlets died before flowering.

The *snp33* phenotype was reminiscent of mutants collectively called disease lesion mimics that show spontaneous cell death in the absence of pathogens. A subclass of these mutants also mimic the cellular and molecular processes involved in plant disease response (Dietrich et al., 1994). Therefore, we tested *snp33* mutants for two easily accessible cellular markers, autofluorescence and callose deposition, which are often correlated with a plant–pathogen interaction. When analyzed in blue light, cells within the lesions showed bright yellow autofluorescence that slightly preceded browning (Fig. 7, G and H). Aniline blue staining to detect callose revealed subcellularly restricted cell wall appositions in mutant cotyledons that were enlarged significantly compared with those observed in wild-type (Fig. 7, I and J).

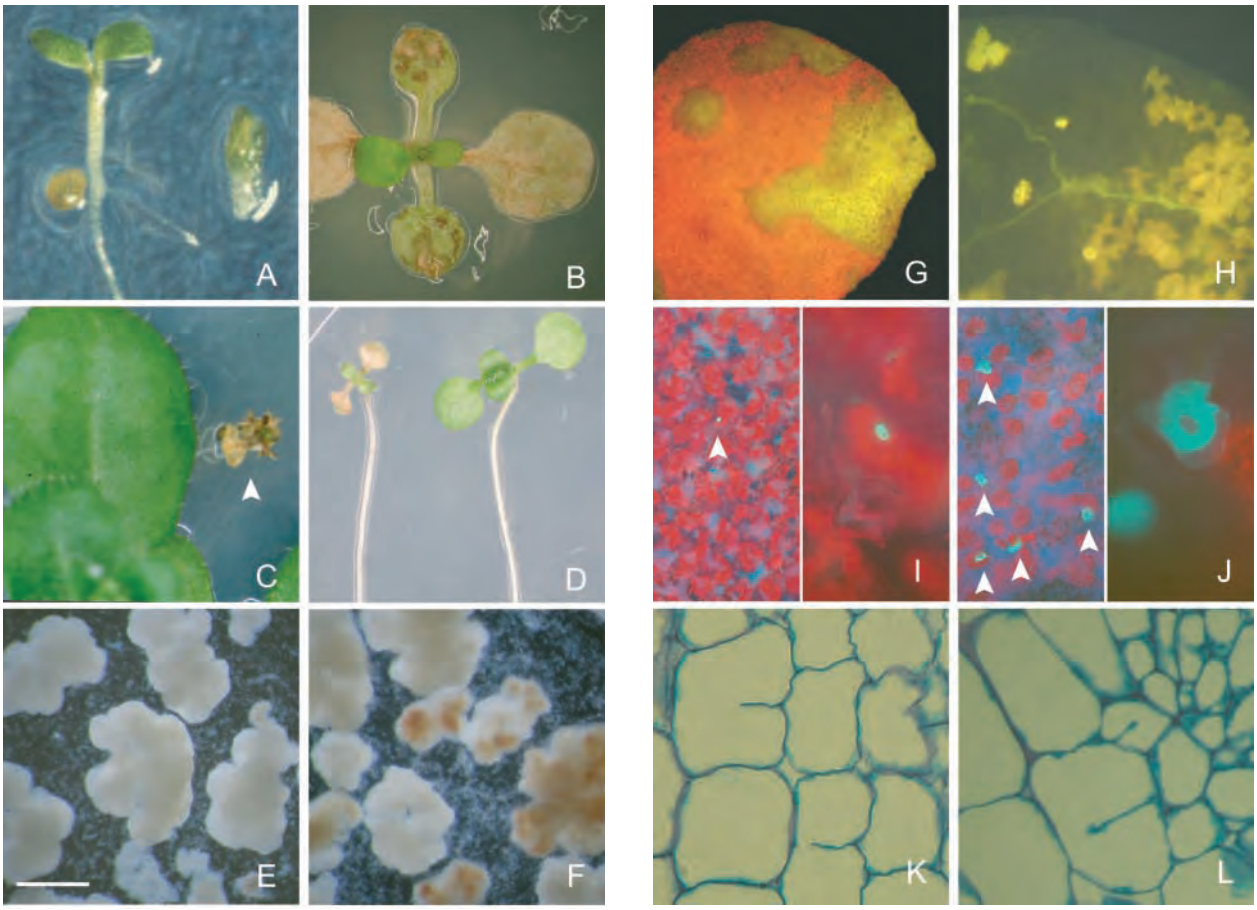

Figure 7. ***snp33* mutant phenotype.** (A–D) *snp33* seedling phenotypes. (A) *keu* mutant seedling (right) next to a 5-d-old wild-type or *snp33* mutant seedling (left). *snp33* mutants were indistinguishable from wild-type at this age. (B) 15-d-old *snp33* seedling with brownish lesions on cotyledons and rosette leaves. Note that the youngest leaves still look normal. (C) Size difference between *snp33* seedling (arrowhead) and wild-type sibling (left) after several weeks on plates. (D) Etiolated *snp33* (left) and wild-type (right) seedlings. After germination and etiolation in the dark, the seedlings were grown in the light to distinguish mutant and wild-type on the basis of brownish lesions. (E and F) Callus culture generated from wild-type (E) and *snp33* seedlings (F). Note brownish speckles in F. (G and H) Yellow autofluorescence of *snp33* mutant tissue observed in blue light. (G) Tip of a young rosette leaf; the red fluorescence is caused by chlorophyll. (H) Detail of a cotyledon after ethanol-acetic acid fixation, which eliminates chlorophyll autofluorescence. Single fluorescing cells can be distinguished easily. (I and J) Aniline blue staining of wild-type (I) and *snp33* (J) cotyledons observed in ultraviolet light. Callose depositions fluoresce bright turquoise, chlorophyll red. Left, overviews, callose depositions marked by arrowheads; right, close-ups. Note that callose depositions are significantly larger in the *snp33* mutant than in wild-type. (K and L) Partially divided cells of a 9-d-old *snp33* mutant seedling seen on 3-μm sections stained with toluidine blue. Details from the petiole of a cotyledon (K) and the hypocotyl/petiole junction (L). Bar: (E and F) 1 mm; (G) 500 μm; (H) 125μm; (I and J, left) 150 μm; (I and J, right) 15 μm; (K and L) 12 μm.

Considering the extreme dwarf phenotype of *snp33* mutant plants, we examined whether growth was affected directly. Dark-grown wild-type seedlings are long and slender due to extreme cell elongation in the hypocotyl. In this assay, *snp33* mutants were indistinguishable from wild-type (Fig. 7 D), indicating that cell expansion is not severely affected. The same seems true for cell division, since a vividly proliferating callus culture could be established from mutant seedlings. *snp33* callus only differed from wild-type by the occasional deposition of brownish material reminiscent of the lesions in the aerial parts of mutant plants (Fig. 7, E and F). This is in contrast to the strong cytokinesis mutants *kn* and *keu* which do not proliferate in callus culture under equivalent conditions.

Although the overall appearance of *snp33* seedlings differed strongly from the seedling phenotype of *kn* or *keu* (Fig. 7 A), the microscopic analysis of toluidine blue–stained sections indicated cytokinesis defects also for *snp33* (Fig. 7, K and L). In *snp33* mutant seedlings, partially divided cells occurred at a higher frequency than in wild-type as determined from longitudinal sections of hypocotyls and cotyledonary petioles. Whereas wild-type on average showed only 0.15 incomplete cell walls per section (n = 150 sections), the corresponding frequency for the *snp33* mutant was 3.6 (n = 163 sections), that is, ~25 times higher. Thus, the mutant analysis suggests that SNP33 is functionally involved in cytokinesis but also in other processes as indicated by the necrotic phenotype.

keu snp33 double mutant analysis

Despite their strong cell division defects during embryogenesis, *kn* and *keu* single mutants develop to the seedling stage (Assaad et al., 1996; Lukowitz et al., 1996; Fig. 7 A). How-

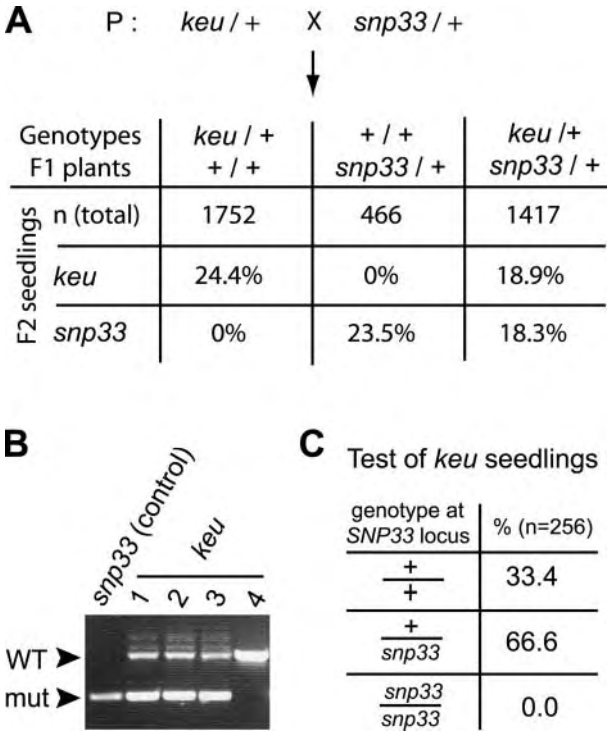

Figure 8. **Analysis of *snp33 keu* double mutants.** (A) A heterozygous *keu* plant was crossed to a heterozygous *snp33* plant, and the segregation rates of *keu* and *snp33* mutant seedlings in the F2 were compared. Note ~5% shortage of each of the mutant phenotypes among the progeny of doubly heterozygous F1 plants compared with those of the singly heterozygous F1 plants. italic, mutant alleles; +, wildtype allele. (B and C) *keu* seedlings from a doubly heterozygous F1 plant were genotyped at the *SNP33* locus. (B) Amplification products of the three-primer PCR used for genotyping (example). The lower band (mut) indicates the presence of the *snp33* mutant allele, and the upper band (WT) indicates the presence of the *SNP33* wild-type allele. snp33 (control), seedling displaying the *snp33* mutant phenotype; *keu*, four *keu* seedlings from a doubly heterozygous F1 plant (1–3, heterozygous at the *SNP33* locus; 4, homozygous wild-type). (C) Determination of the *SNP33* genotype of 256 *keu* seedlings from a doubly heterozygous F1 plant.

ever, cytokinesis is blocked completely in the *kn keu* double mutant, resulting in single-celled multinucleate embryos (Waizenegger et al., 2000). Therefore, we examined whether a combination with *keu* might also enhance the weak cytokinesis defects seen in *snp33* mutants. Among the seedling progeny of a selfed *keu*/+;*snp33*/+ plant, the frequencies of the two single mutant phenotypes, *keu* and *snp33*, were reduced by ~5% compared with single mutant sister lines (Fig. 8 A). In addition, no novel seedling phenotype was observed, suggesting that the *keu snp33* double mutants died before germination. Alternatively, disregarding the observed segregation ratio, double mutant seedlings might resemble *keu* seedlings if their growth was arrested before the onset of *snp33* necrosis. To distinguish between these possibilities, we tested the *SNP33* genotype of *keu* seedlings from a doubly heterozygous mother plant. None of the 256 *keu* seedlings analyzed by PCR were homozygous for *snp33,* whereas *keu* seedlings heterozygous for *snp33* occurred at the expected frequency (Fig. 8, B and C). Thus, *keu snp33* double mutants indeed did not reach the seedling stage. A comparable analysis for *snp33* in combination with *kn* was hampered by the variable germination rate of *kn* mutants and an unreliable PCR on the very small *kn* seedlings. However, we obtained preliminary data that indicate a similar effect as for the *keu snp33* double mutant (unpublished data).

SNP33 is a member of a small protein family in *Arabidopsis*

No SNP33 protein was detected in *snp33* mutant tissue (Fig. 2) even after overexposure of the Western blots, indicating that the mutant represents a complete loss of function. Thus, residual gene function does not account for the rather weak cytokinetic phenotype of *snp33* mutants. Sequencing of the *Arabidopsis* genome identified two additional SNAP25 homologous genes, *AtSNAP29* (AGI-ID: At5g07880) and *AtSNAP30* (AGI-ID: At1g13890). The deduced proteins show ~62% identity to AtSNAP33 and 52% identity among each other (Fig. 9), which raised the issue of functional redundancy. Therefore, we tested their ability to interact with the KN syntaxin in the yeast two-hybrid assay (Fig. 10). All three SNAP25 homologues of *Arabidopsis* interacted with the KN cytoplasmic region, suggesting that cytokinetic defects of *snp33* single mutants may be weakened by activities of the other SNAP25 homologues.

Discussion

Our search for interactors of the cytokinesis-specific syntaxin KN identified the t-SNARE AtSNAP33 (SNP33), a member of a small family of SNAP25 homologous proteins in *Arabidopsis*. We characterized *SNP33* by expression pattern and subcellular localization of the protein and by phenotypic and genetic analysis of a loss-of-function mutant. Our data suggest that SNP33 is involved in several processes, including cytokinesis, and that its function in cytokinesis may be replaced partially by other SNAP25 homologues.

Membrane association of AtSNAP33

In cell fractionation experiments, SNP33 was tightly associated with membranes from which it was only removed by detergent thus behaving like an integral membrane protein. How SNP33 associates with membranes is an open question. Unlike most SNAREs, SNAP25-type proteins lack a membrane-spanning domain, but some SNAP25 homologues attach to membranes via a lipid membrane anchor (Steegmaier et al., 1998). A conserved cysteine cluster has been shown to be palmitoylated and responsible for initial membrane association of SNAP25 (Veit et al., 1996). However, this cluster is not present in SNP33. It is also missing in mammalian SNAP29 and the yeast SNAP25 homologues Sec9p and Spo20p, but in contrast to SNP33, Sec9p and SNAP29 can be extracted from membranes with high pH (Brennwald et al., 1994; Steegmaier et al., 1998), suggesting that they do not carry a lipid anchor at all. Whether SNP33 is lipid modified at its only cysteine at position 119 or at another residue remains to be investigated.

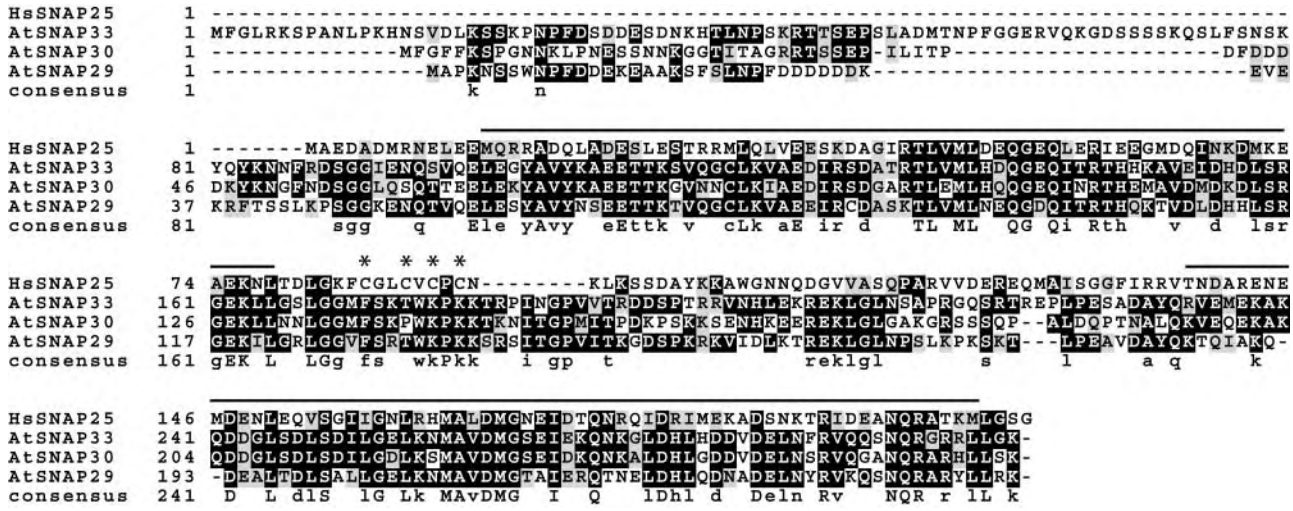

Figure 9. **Protein sequence alignment of *Arabidopsis* SNAP25 homologues.** Human SNAP25 was aligned with the three SNAP25 homologues of *Arabidopsis*. The lines above the sequences indicate the α-helical regions possibly involved in SNARE complex formation. The conserved cysteine residues of HsSNAP25 involved in palmitoylation are marked by asterisks. Consensus: capital letters, residues conserved in all four proteins; small letters, residues conserved among the three *Arabidopsis* homologues. The sequence data are available from GenBank/EMBL/DDBJ under accession nos: HsSNAP25, NP_003072; AtSNAP29, CAB62600; AtSNAP30, AAF79396; AtSNAP33, BAB10383.

Subcellular localization studies indicated that SNP33 is localized predominantly at the plasma membrane and cell plate of dividing cells. Additional weaker labeling in the cytoplasm may represent cytosolic or endomembrane-bound protein. In support of the latter, BFA treatment, which leads to an agglomeration of endomembranes, resulted in an accumulation of SNP33 label in distinct patches within the cell that were marked also by the transmembrane protein PIN1. The SNP33-labeled endomembranes may be transit compartments to the plasma membrane or endosomes.

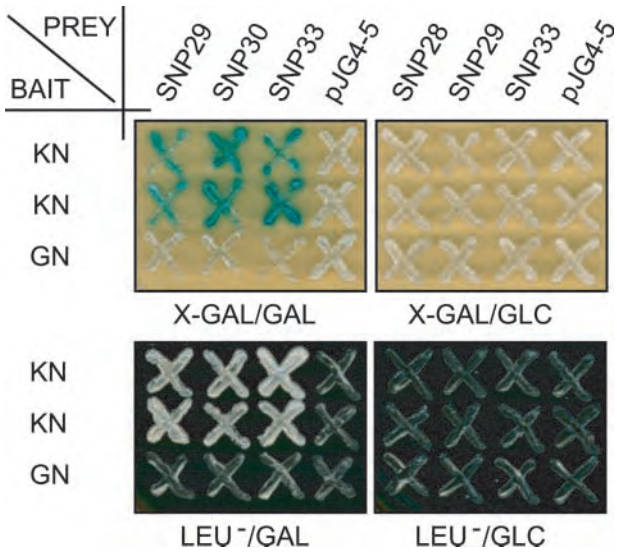

Figure 10. **Interaction of KN with different *Arabidopsis* SNAP25 homologues.** Using the two-hybrid system, the cytoplasmic domain of KN was tested for interaction with full-length constructs of the *Arabidopsis* SNAP25 homologues. GNOM (GN) bait and empty prey vector (pJG4-5) were used as negative controls. Two independent clones are shown when KN was used as the bait. Interaction was tested in the color assay on X-gal containing plates (top, X-Gal) and in the growth assay on medium lacking leucine (Leu⁻, bottom). Interaction was only observed upon induction of the prey constructs with galactose (left, GAL) and not on glucose-containing plates (right, GLC).

A role for SNP33 in cytokinesis

We demonstrated that SNP33 interacts with the cytokinesis-specific syntaxin KN under different experimental conditions, implying a role for SNP33 in cell division. In addition, both proteins colocalized at the forming cell plate, which strongly suggests that the interaction observed in vitro reflects the situation in vivo. Functional data also support a role for SNP33 in cytokinesis. The phenotypic analysis of *snp33* mutant plants revealed incomplete cell walls as expected for cytokinesis defects, and double mutants of *snp33* and the strong cytokinesis mutant *keu* displayed synthetic embryo lethality, suggesting that both proteins promote the same process. Interestingly, synthetic embryo lethality has also been observed for *kn keu* double mutants (Waizenegger et al., 2000), whereas both single mutants develop until the seedling stage. Both, SNP33 and KEU are members of small gene families in *Arabidopsis*, which suggests a simple mechanistic interpretation of the double mutant data. In this scenario, KEU function can in part be fulfilled by a KEU homologue if SNP33 is present, and SNP33 can be substituted by a SNP33 homologue if KEU is present. However, the two substitutes are fully incompatible with one another such that a functional pathway is blocked completely in the *keu snp33* double mutant. This does not imply necessarily that SNP33 and KEU interact directly, although Sec1p has been shown to bind to the core fusion complex in yeast (Carr et al., 1999).

Implications for a SNARE complex in cell plate formation

In plant cytokinesis, vesicle fusion plays a prominent role in the formation and lateral expansion of the cell plate. Although the

identification of the cytokinesis-specific syntaxin KN demonstrated the involvement of a SNARE complex in cell plate formation, its exact composition has not been resolved. Since KN lacks orthologues in nonplant organisms, it was conceivable that the cytokinetic SNARE complex might have a unique composition. Our characterization of SNP33 suggests that the cytokinetic SNARE complex consists of a syntaxin and a SNAP25 homologue thus resembling trimeric SNARE complexes acting at the plasma membrane in yeast and mammals, in contrast to tetrameric complexes found preferentially on endomembranes (Fukuda et al., 2000). This similarity suggests that cell plate formation is a variant of exocytosis. An analogous situation has been reported for yeast where related SNARE complexes mediate exocytosis at the plasma membrane of vegetative cells and membrane fusion during sporulation, a process described as similar to plant cytokinesis (Neiman, 1998). In yeast, both SNARE complexes use the same syntaxins and synaptobrevins but employ functionally distinct SNAP25 homologues, Sec9p in vegetative cells as opposed to Spo20p in sporulation (Neiman et al., 2000). By contrast, in *Arabidopsis* the syntaxin KN is a specific component of the cytokinetic SNARE complex, whereas the SNAP25 homologue SNP33 appears to be shared with other SNARE complexes (see below).

In summary, we propose that the specific KN syntaxin contributes one helix and the more general SNP33 adds two helices to the cytokinetic fusion complex. Assuming that the number of helices in SNARE core complexes is invariant, it will be important to determine what protein, general or specific, contributes the fourth helix to this bundle.

Other roles for SNP33

In plants, no SNARE complexes at the plasma membrane have been described, although candidates for plasma membrane syntaxins have been proposed (Sanderfoot et al., 2000). Because of its subcellular localization and its ubiquitous cell cycle–independent expression, SNP33 is a likely component of a general SNARE complex at the plant plasma membrane in addition to its role in cytokinesis. This assumption is supported by the finding that SNP33 binds at least one syntaxin other than KN in the yeast two-hybrid assay (unpublished data). The mammalian SNAP25 homologues, especially SNAP29, also seem to be binding partners for a variety of syntaxins (Steegmaier et al., 1998). Furthermore, some features of the *snp33* lack-of-function mutant, such as the formation of necrotic lesions, autofluorescence, and enhanced localized callose deposition, cannot be related easily to defects in cytokinesis but are rather reminiscent of a heterogeneous class of mutants collectively called disease lesion mimics (Dietrich et al., 1994). Some of these mutants are related to pathogen response, whereas others are perturbed in cellular metabolism, with lesions occurring at specific developmental stages (Hu et al., 1998), as also observed for *snp33*. Thus, SNP33 function may become especially important at a particular developmental stage, most likely due to a lack of redundancy (see below). Although mutations in a growing number of genes result in disease lesion mimicry, *snp33* is the first to affect vesicle fusion. How the latter defect causes the lesion phenotype remains to be investigated.

Specificity and redundancy of SNAP25 homologues

The ubiquitous expression of SNP33 contrasts with its more restricted requirement as revealed by the mutant phenotype, indicating that its function is nonessential in some contexts. One possible explanation would be that other proteins perform overlapping functions. We envision a scenario in which there is perfect redundancy of SNP33 function in a field A, such as embryo viability, partial redundancy in a field B, such as cytokinesis, and insufficient redundancy in a field C, which finally causes death of the *snp33* mutant. AtSNAP29 and AtSNAP30 might substitute for SNP33 in cell division, since they interacted also with KN in the yeast two-hybrid assay, and the corresponding mRNAs were represented in cDNA libraries made from proliferating tissues.

Sequence analysis of the *Arabidopsis* genome revealed extensive segmental duplication, possibly reflecting an ancient tetraploidization event (The *Arabidopsis* Genome Initiative, 2000). For example, AtSNAP29 and AtSNAP33 are located in a duplicated region of ~100 kb (K. Mayer, personal communication), indicating that once in plant evolution they had the same function. Derivative copies of an ancestral gene may have retained the original regulation and function or may have specialized. A striking example of overlapping function involving three genes is found in flower development of *Arabidopsis*. In the triple mutant *sepallata 1/2/3*, organ identity is changed to sepals in all whorls of the flower, whereas each single mutant displays only a very subtle phenotype (Pelaz et al., 2000). Redundancy might also be valid for the SNAP25 family at least in certain processes, such as cytokinesis. By contrast, *Arabidopsis* syntaxins seem to be more unique. In addition to *kn*, which shows severe cytokinetic aberrations already early in embryonic development (Lukowitz et al., 1996), four other *Arabidopsis* syntaxin mutants have been described (*syp21-2*, *syp22*, *syp41*, and *syp42*) (Sanderfoot et al., 2001). Homozygous mutant progeny could be identified for none of the latter, implying that each has essential functions.

Conclusion

Our data suggest that SNP33, the first SNAP25 homologue functionally characterized in plants, cooperates with the KN syntaxin and the KEU Sec1 homologue during cell plate formation. Further analysis will reveal to what extent SNP33 is involved in SNARE complex formation at the plasma membrane and how SNP33 function relates to the observed disease lesion mimic phenotype. In addition, the SNAP25 family of *Arabidopsis* will be an excellent model to study the extent and significance of functional redundancy among members of small gene families that are involved in basic cellular processes.

Materials and methods

Yeast two-hybrid assay

We used the lexA-based interaction-trap system as described in Ausubel et al. (1995). An *Arabidopsis* cDNA library generated from young siliques (Grebe et al., 2000) was screened for KN-interacting proteins using the yeast strain EGY-48 and the lacZ reporter pSH18-34. KN cytoplasmic domain (amino acids 1–287) was amplified from cDNA and cloned into the bait vector pEG202 using restriction sites added by PCR (EcoRI/BamHI). In the screen, 1.8×10^6 primary transformants were replated by 2×10^7 colonies on leu^- medium; 1041 clones were grown after 4 d, and 96 of these also showed galactose-dependent activation of the lacZ reporter and were further analyzed. Restriction digests of the prey inserts amplified by PCR

allowed grouping into 24 classes, and sequencing showed that three classes represented clones of different length derived from the gene *AtSNAP33 (SNP33).* To test for interaction of full-length SNP33, AtSNAP30, and AtSNAP29 with KN, the prey vector pJG4-5 was modified. The internal BamHI site was destroyed, and a new BamHI site was inserted in frame into the EcoRI site of the polylinker. The SNAP25 homologues were amplified from cDNA and cloned via BamHI/XhoI into the modified pJG4-5. Restriction sites were added by PCR. The bait construct lexA-$GNOM_{18–45}$ (Grebe et al., 2000) was used as a negative control.

Plant material and growth conditions

The *snp33* mutation was induced in *Arabidopsis thaliana* ecotype Wassilewskija. The *keu* allele AP77, ecotype Landsberg *erecta,* was used in the double mutant analysis.

Plants were grown as described previously (Mayer et al., 1991). To generate callus cultures, 30–50 seedlings were transferred into 100 ml liquid medium (4.3 g/liter MS salts, 0.5 g/liter MES, 3% sucrose, 1× Gamborg's B5 vitamins [Duchefa], 1 mg/liter 2,4-D and 0.25 mg/liter kinetin, pH 5.8) and incubated at 20°C in constant light with agitation. When sufficient callus material had developed, the cultures were split into two, once a week, and supplemented with new medium. The suspension culture used in GST pulldown experiments was a kind gift of the John Innes Centre (Norwich, UK) and was propagated as described previously (Fuerst et al., 1996).

For the in vitro root culture, 10-d-old plantlets were transferred to liquid medium (0.46% MS salts, 0.018% KH_2PO_4, 0.02% myo-inositol, 0.001% thiamin, 0.0001% pyridoxin, 0.0001% biotin, 0.0002% glycin, 0.0001% nicotinic acid, 0.00005% folic acid, 3% sucrose, pH 5.8) and incubated for 3 wk in darkness with slow agitation. Then, the roots were cut, transferred to the same liquid medium, and grown in the dark. Every 4 wk, the roots were divided in four parts and transferred to fresh medium.

Preparation of microsomes and solubilization of SNP33

5 g of roots cultivated in vitro were homogenized on ice with mortar and pestle in 4 ml of 50 mM 3-morpholinopropanesulfonic acid buffer, pH 7.6, containing 0.5 M sorbitol, 10 mM EGTA, 2.5 mM potassium metabisulfite, 4 mM salicylhydroxylamic acid, 5% polyvinylpyrrolidone, and 1% BSA. The mixture was filtered through miracloth and centrifuged at 8,000 *g* for 15 min at 4°C. The supernatant was centrifuged at 100,000 *g* for 30 min at 4°C. To test for solubilization of SNP33, the 100,000 *g* pellet was resuspended in 10 mM phosphate buffer, pH 7.6, containing 0.5 M sorbitol. The suspension was divided in five aliquots, which contained either 2 M urea, 1% sodium dodecyl sulfate, 1 M NaCl, 0.1 M Na_2CO_3, pH 11.0, or 10 mM phosphate buffer, pH 7.6, and incubated for 30 min at room temperature. Half of each aliquot was kept, which represents the total membrane, and the other half was centrifuged at 100,000 *g* for 1 h at 4°C. Supernatant and total membrane were analyzed by SDS-PAGE and Western blots.

In vitro binding of SNP33 and KN

Full-length *SNP33* was cloned into pGEX-4-T1 (Amersham Pharmacia Biotech) via BamHI/XhoI restriction sites added by PCR. The cytoplasmic domain of KN (amino acid 1–284) was cloned into pGEX-4-T1 via addition of EcoRI/XhoI restriction sites. GST pulldown experiments were performed as described previously (Grebe et al., 2000).

Immunofluorescence and Western blot analysis

Whole-mount immunofluorescences, preparation of protein extracts, and Western blots were performed as described in Lauber et al. (1997). The anti-KN serum (Lauber et al., 1997) was used at 1:6,000 for Western blots and at 1:4,000 for immunofluorescence analysis. The anti-PIN1 serum (Gälweiler et al., 1998) was used at 1:200. Myc-AtSNAP33 was detected with the mouse monoclonal anti–c-myc antibody 9E10 (Santa Cruz Biotechnology, Inc.) at 1:250. The anti-SNP33 serum was used at 1:4,000 and was generated as follows: a 6×His tag was added to the NH_2 terminus of SNP33, leading to the sequence MRGSHHHHHH followed by amino acids 8–300 of SNP33. Recombinant His-SNP33 was expressed in *Escherichia coli* XL-1 and purified via nickel affinity chromatography under denaturing conditions following the manufacturer's instructions (QIAGEN). The eluted protein was purified further via SDS-PAGE. A band of the correct size was cut from a gel stained in 0.3 M $CuCl_2$ for 10–20 min. After destaining by several changes of 0.25 M Tris, pH 8.0, 0.25 M EDTA, the protein was eluted from the squashed gel matrix in PBS, pH 7.3, and concentrated using a centrifugal concentrator (Macrosep, 10K9; Pall Filtron). Rabbit immunization was performed as described in Lauber et al. (1997). Unpurified serum of the third bleeding was used in all experiments. Secondary antibodies were used at the following dilutions: HRP-conjugated anti–rabbit IgG at 1:2,000 for Western blots (Boehringer) and anti–rabbit-FITC at 1:250, anti–rabbit-Cy3™ at 1:600, and anti–mouse-Cy3™ at 1:600 for immunofluorescence analysis (Dianova). Whole-mount immunofluorescences were analyzed using a confocal laser scanning microscope (Leica) and the Leica TCS-NT software.

T-DNA insertion lines screen

For T-DNA structure and experimental procedures used in the insertion line screen, see Bechtold et al. (1993). The T-DNA insertion line of *SNP33* (originally called EFS396) was detected in the DNA pool 307A using the *SNP33* genomic primer GMS25BW5 (5′-AGTCCAAACCTCCACTCTCT-GATAAGC-3′) and the T-DNA primer TAG3 (5′-CTGATACCAGACGT-TGCCCGCATAA-3′).

Plant transformation constructs and plant transformation

For cloning the *SNP33* genomic rescue constructs, we modified the plant transformation vector pGPTV-HPT (Becker et al., 1992) by deleting the GUS gene via an EcoRI/XbaI digest and religating the blunted vector (pGPTV-HPTmod). The resulting polylinker was EcoRI-XbaI-SalI-HindIII. The genomic fragments of *SNP33* were derived from the P1 clone MAF19 (*A. thaliana*, ecotype Columbia). Rescue construct A (Fig. 4) is a 5-kb EcoRI fragment cloned into pGPTV-HPTmod. For rescue construct B (Fig. 4), a 4.8-kb EcoO109I (blunted)/XhoI fragment was ligated into an EcoRI (blunted)/SalI cut pGPTV-HPTmod-vector. The myc tag was inserted into an unique SalI site of fragment B using the overlapping oligos MYC-sense (5′-TCGATGGAGCT*GAGCAAAAGCTTATTTCT-GAGGAGGATCTTCTT*GCTGGATCAG-3′) and MYC-antisense (5′-TCGACTGATCCAGC*AAGAAGATCCTCCTCAGAAATAAGCTTTTGCTC*AG-CTCCA-3′). Nucleotides coding for the c-myc epitope are written in italics. For the pSNP33–GFP construct, a 2.1-kb promotor fragment of SNP33 (Fig. 4, fragment C, ecotype Landsberg *erecta*) was amplified using the primers 5′-GGAGATATCGGAGGAAGAATGCAAGC-3′ and 5′-GGAGGATCCACAAAAGGAACACTTGG-3′, fused with the cDNA of mGFP5-ER (Haseloff et al., 1997), and ligated into the binary vector pBin19. Expression of the *pAtSNAP33–GFP* transgene was analyzed with the Leica DM R microscope, and pictures were taken with the CCD camera AxionCam (ZEISS) and analyzed with the software Axion Vision 2.05. The floral-dip method (Clough and Bent, 1998) was used for *Agrobacterium*-mediated plant transformation.

Determination of the *SNP33* genotype

The genotype of plants from rescue experiments was determined by PCR using the *SNP33* genomic primers GMS25FW1 (5′-TCCATCTTCTTCT-TCACGGACTCCAC-3′) and GMS25BW2 (5′-GAGTGGTTCTCGGGTTCT-TGATTGTC-3′). This PCR yielded only a product if no T-DNA was present in the *SNP33* gene. To differentiate between the endogenous Wassilewskija wild-type allele and the Columbia rescue construct, a SpeI restriction polymorphism was used. Only the Columbia fragment was cut. Plants from the rescue experiment using the myc-SNP33 construct were analyzed using the primer pair GMS25FW4 (5′-GCTAGATCCTGGGCTTTCGATTTG-3′) and 48SP2 (5′-GAACCGACTGGTTTTCAATACCACC-3′), which only gave a product if no T-DNA was inserted in *SNP33*. The myc-tagged fragment could be distinguished from the endogenous wild-type *SNP33* allele by a 54 bp length difference. To genotype *keu* seedlings for SNP33 in the double mutant analysis, a three-primer PCR was used, involving the SNP33 genomic primers GMS25FW4 and 48SP2 and the T-DNA primer TAG3. A 340-bp fragment resulted from the presence of the T-DNA insertion, and a 730-bp band indicated a *SNP33* allele without the insertion (Fig. 8).

Phenotypic analysis

Plantlets were analyzed using a Leica MZ125 binocular and a ZEISS microscope. Pictures were taken with a digital camera (Coolpix 990; Nikon). Autofluorescence was analyzed by observation in blue light (450–490 nm). Callose deposition was monitored by staining with aniline blue (45 min, 0.5 mg/ml in water) and observation in ultraviolet light. For sectioning, seedlings were fixed overnight in 4% paraformaldehyde in PBS, dehydrated via an ethanol series, and embedded in LR-White resin (London Resin Company, Ltd.). 3-μm sections were cut using a Leica microtome (Supercut 2065) and stained for 20 s with 0.1% toluidine blue, 0.1% borate. Stained sections were embedded in Eukitt (Kindler GmbH).

Other techniques

Molecular techniques were performed according to Ausubel et al. (1995) or the manufacturer's protocol. All constructs cloned via a PCR-based step were

confirmed by sequencing using the ABI PRISM BigDye Terminator cycle sequencing kit and the ABI sequencer 310 (Applied Biosystems). Sequence analysis was carried out with Vector NTI™ (Informax). Alignments were done with the ClustalW algorithm (Thompson et al., 1994). Images were processed with Adobe Photoshop® 6.0 and Adobe Illustrator® 9.0 software.

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The expression of the t-SNARE AtSNAP33 is induced by pathogens, mechanical stimulation, and UV irradiation

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Abbreviations: dpi, days post-inoculation; NSF, N-ethylmaleimide-sensitive factor; PR proteins, pathogenesis-related proteins; SA, salicylic acid; SNAP, soluble NSF attachment factor; SNARE, SNAP receptor; *TCH* genes, touch-genes

Abstract

The fusion of vesicles in the secretory pathway involves the interaction of t-SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) on the target membrane and v-SNAREs on the vesicle membrane. AtSNAP33 is an *Arabidopsis thaliana* homologue of the neuronal t-SNARE SNAP-25 involved in exocytosis and is localized at the phragmoplast and at the plasma membrane. Herewith, the expression of AtSNAP33 was analysed after different biotic and abiotic stresses. The expression of AtSNAP33 increased after inoculation with the pathogens *Peronospora parasitica*, isolates NOCO and EMWA, *Plectosporium tabacinum* and *Pseudomonas syringae* pv *tomato* DC3000 carrying or not the avirulence gene *avr*Rpt2. The expression of *PR1* transcripts which encode the secreted pathogenesis-related protein 1 (PR1) also increased after inoculation with these pathogens and the expression of *AtSNAP33* preceeded or occurred at the same time as the expression of *PR1*. AtSNAP33 was also expressed after pathogen inoculation in *npr1* plants which do not express PR1 as well as in *cpr1* plants which overexpress PR1 in the absence of a pathogen. The level of AtSNAP33 increased after inoculation with *P. parasitica* in the *nahG* plants, and *eds5* and *sid2* mutants which are unable to accumulate SA after pathogen inoculation indicating that SA was not necessary for the induction of the expression of AtSNAP33 in inoculated leaves. However, it was sufficient for this induction since the expression of AtSNAP33 increased after treatment with SA. It increased as well after treatment with BTH, an inducer of defense reactions. AtSNAP33 was also expressed in systemic non inoculated leaves of plants inoculated with *P. syringae.* This systemic expression was abolished in *nahG* plants indicating that it required SA. Thus, the expression of AtSNAP33 after pathogen attack is regulated by SA-dependent and SA-independent pathways. UV-C irradiation led to an increase of AtSNAP33 and *PR1* transcripts. After mechanical stimulation and wind, the levels of *AtSNAP33*

transcripts increased within 15 min and were back to control levels after 90 min. These changes were similar after mechanical stimulation of the *glabrous1* mutant which bears almost no trichomes, indicating that the increased expression was not due to breakage of the trichomes, and after wounding.

Introduction

The plant secretory pathway comprises different organelles including the endoplasmic reticulum, the Golgi apparatus, the plasma membrane and the vacuole. Proteins destined to the extracellular space or the vacuole enter the secretory pathway at the endoplasmic reticulum and are then transported through the Golgi apparatus. At the trans-Golgi network, secreted proteins are sorted from vacuolar proteins and packaged into secretory vesicles. Transport between the organelles of the secretory pathway occurs by budding of vesicles from a donor membrane and fusion with an acceptor membrane. Exocytosis is the fusion of secretory vesicles with the plasma membrane, permitting the release of their content outside the cell. The fusion of vesicles involves the interaction of v-SNAREs (vesicle SNAP receptors) localized on the vesicle membrane and t-SNAREs localized on the target membrane (Rothman, 1996; Hay and Scheller, 1997). A four-helical bundle of SNAREs is formed. One helix of this SNARE complex is provided by a v-SNARE and three helices by t-SNAREs which always include a member of the syntaxin family contributing one helix. The remaining two helices are contributed either from a single SNAP-25-like protein or from two separate t-SNARE light chains. Trimeric SNARE complexes have been described for the fusion of vesicles at the plasma membrane whereas endomembrane fusion processes involve tetrameric SNARE complexes (Fukuda, 2000). Two cytosolic proteins, general components, α-SNAP (soluble NSF-attachment protein) and NSF (N-ethymaleimide-sensitive factor) disassemble this complex after fusion. The

machinery of membrane fusion is highly conserved among eukaryotes (for reviews, see Blatt *et al.*, 1999, Jahn and Südhof, 1999). Several plant SNAREs have been isolated and the sequence of the Arabidopsis genome contains many SNAREs (for a review, see Sanderfoot *et al.*, 1999, 2000). The function of most of these proteins remains to be demonstrated. However, they often show a high degree of conservation with their yeast or mammalian counterparts. Thus, they may have similar functions in the cell.

Plants respond to bacterial, fungal and viral pathogen attack by the synthesis of several pathogenesis-related (PR) proteins including PR1 (van Loon and van Strien, 1999). Many of them are synthesized at the endoplasmic reticulum and transported to the extracellular space or the vacuole by the secretory pathway. For example, infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv *tomato* DC 3000 leads to the induction of the expression of PR1, PR2 (a β-1,3-glucanase) and PR5 (a thaumatin-like protein) which are secreted to the extracellular space (Uknes *et al*., 1992). The accumulation of the signalling molecule salicylic acid (SA) correlates with the expression of PR proteins and resistance (Malamy *et al*., 1990, Métraux *et al*., 1990, Yalpani *et al*., 1991). Transgenic tobacco and *A. thaliana* plants that express a bacterial *nahG* gene encoding a SA hydroxylase degrade SA to catechol (Gaffney *et al*., 1993, Delaney *et al*., 1994) and express very low amounts of PR proteins after pathogen attack (Delaney *et al*., 1994). In the *A. thaliana eds5* and *sid2* mutants there is no accumulation of SA after pathogen attack and a strong reduction of PR1 expression but PR2 and PR5 are still expressed (Nawrath and Métraux, 1999).

Many plants respond to mechanical stimulation such as touch and wind by a reduced growth and become stockier. These developmental changes called thigmomorphogenesis make the plant more resistant to environmental challenges such as blasts of wind. The effect of mechanical strain on plant growth has been well documented (Jaffe and Forbes, 1993, Mitchell and Myers, 1995). However, the molecular mechanisms underlying these effects are not well understood. The

expression of several genes from *A. thaliana* has been shown to be regulated by mechanical stimulation. They include the so-called *TCH* genes (Braam and Davis, 1990, Braam *et al*., 1997) and several genes encoding protein kinases (Mizoguchi *et al*., 1996). *TCH1*, *TCH2* and *TCH3* encode Ca^{2+}-binding proteins and *TCH4* encodes a xyloglucan endotransglycosylase (Xu *et al*., 1995). Genes responding to mechanical stimulation have also been identified in other plant species and include calmodulins in potato (Takezawa *et al*., 1995), mung bean (Botella *et al*., 1995), tomato (Depège *et al.*, 1999) and tobacco (van der Luit *et al*., 1999), ACC synthases in mung bean (Botella *et al*., 1995) and tomato (Tatsuki and Mori, 1999) and a lipoxygenase in wheat (Mauch *et al*., 1997).

We have previously characterized AtSNAP33, a homologue of the t-SNARE SNAP-25, in *Arabidopsis thaliana* (Heese *et al.*, 2001). AtSNAP33 was localized at the plasma membrane and at the forming cell plate. It interacts with the syntaxin KNOLLE localized at the cell plate (Heese *et al*., 2001) and Nt-Syr1, a tobacco syntaxin localized at the plasma membrane (Kargul *et al*., 2001). The null mutant *atsnap33* develops large necrotic lesions on cotyledons and rosette leaves as well as cytokinetic defects and eventually dies before flowering. We postulate that AtSNAP33 could be involved in diverse membrane fusion processes including exocytosis and the formation of the cell plate.

Here, we show that the expression of AtSNAP33 was induced after inoculation with pathogens and occurs in inoculated leaves and in systemic uninoculated leaves. The expression was compared with the expression of PR1, taken as a marker for a gene encoding a pathogenesis-related protein. The expression was studied as well in *nahG* transgenic plants and *eds5*, *sid2*, *npr1* and *cpr1* mutants. The expression of AtSNAP33 was also induced by mechanical stimulation, wind, and UV irradiation.

Material and methods

Plant material, mechanical stimulation, wind treatment, wounding and UV irradiation

A. thaliana accession Col-0 plants were grown in growth chambers under short day conditions (12h light/12h dark) at 20°C. For the mechanical stimulation, leaves of 4 week-old plants were gently rubbed with ungloved fingers 10 times over the entire leaf surface, unless otherwise stated. For wind treatment, 4 week-old plants were exposed to a hair drier at a distance of 20 cm during 1 minute. Plants were wounded by squeezing the leaves between flat-bladed pliers at two different sites. For UV-C irradiation, plants were placed at 30 cm of an UV lamp (254 nm,) for 20 min. Then, they were placed under continuous light in a growth chamber for 17 h before collection of the leaves. The *npr1* and *cpr1* mutants were a kind gift of Dr. X. Dong (Duke University, Durham, North Carolina, USA) and the *eds5* and *sid2* mutants were a kind gift of Dr. C. Nawrath (University of Fribourg, Fribourg, Switzerland).

Inoculation with *Peronospora parasitica* isolates NOCO and EMWA, *Pseudomonas syringae* pv *tomato* DC 3000 and *P. syringae* pv *tomato* DC 3000 *avr Rtp2* and *Plectosporium tabacinum*

3 week-old *A. thaliana* plants, grown in pots (20-30 plants per pot) were inoculated by spraying with a conidial suspension (10^5 - 10^6 conidia/ml tap water) of *Peronospora parasitica* isolate NOCO or EMWA prepared as described (Mauch-Mani and Slusarenko, 1994). After the treatment, the plants were kept in a growth chamber under short day conditions, high air humidity and at 18°C. The control

plants were treated with water.

Pseudomonas syringae pv *tomato* DC 3000 *avr Rpt2* and *P.syringae* pv *tomato* DC 3000 avr *Rpt2* were grown overnight in LB liquid medium (Miller's Luria broth base, Sigma) at 28°C. A dilution of 2 x 10^8 bacteria/ml water was introduced by infiltration with a syringe through the lower side of leaves of 4-5 week-old *A. thaliana* plants grown as one plant/pot. Five leaves per plant were inoculated, subsequently harvested and analysed. The control plants were infiltrated with water. After the inoculation the plants were kept in the growth chamber until collection of the inoculated leaves, respectively the systemic leaves.

Plectosporium tabacinum (a kind gift of Dr. B Mauch-Mani, University of Neuchâtel, Neuchâtel, Switzerland) was kept in Petri plates on potato-dextrose-agar medium (Difco laboratories). To obtain conidia, the surface was scraped with an inoculation loop and the conidia were suspended in water. Conidia were counted under a microscope and a suspension of 2 x 10^6 conidia/ml was prepared in water and sprayed onto 3 week-old plantlets grown in pots containing 20-30 plantlets/pot.

Chemical treatments

Three week-old *A. thaliana* Col-O plants grown in pots with 20-30 plants per pot were soil-dreched with a final concentration of 0.33 mM benzo(1,2,3)thiadiazole-7 carbothioic acid S-methyl ester (BTH, Bion 50 WG, Syngenta) and 330 μM or 1 mM salicylic acid and for controls, plants were treated with wetting powder or H_2O, respectively.

RNA preparation, RNA blot analysis and probes

For RNA extraction, plant tissues were harvested, frozen and pulverized in liquid

nitrogen. One volume of 2 M Tris pH 8 containing 0.5 M EDTA pH 8 and 20% SDS (v:v:v; 1:2:1) at 95 °C was added, followed by 1 volume of saturated phenol:chloroform:isoamyl alcohol (v:v:v; 25:24:1) at 40 °C. The two phases were separated by centrifugation, the aqueous phase containing the RNA was mixed with 1 volume of chloroform and separated by centrifugation. The RNA was precipitated overnight with 1 volume of 6M LiCl at 4°C, washed with 70% ethanol and resuspended in water. For RNA blot analysis, 6 μg of total RNA were applied on a 1% formaldehyde-agarose gel, separated and transferred to a nylon membrane (Hybond-N Amersham) as decribed by Sambrook et al. (1989). The membranes were hybridized overnight at 65°C with a DNA probe made by random primed labeling in the presence of -32P dCTP with the RadPrimed DNA Labeling System according to manufacturer's instructions (Life Technologies). The membranes were washed subsequently for 5 min with 2 X SSC containing 0.1% SDS and then 3 times for 20 min with 0.2 X SSC containing 0.1% SDS at 65°C before exposing to X-Omat film (Kodak).

Protein extraction and protein blot analysis

A.thaliana tissues were harvested, frozen and ground at 4°C with a mortar and a pestle in 2 ml of SDS-PAGE sample buffer per g fresh weight. The homogenate was centrifuged for 10 minutes at 14 000 rpm in an Eppendorf 5415C centrifuge at 4 °C and the supernatant was collected.

Proteins were fractionated on a 13% SDS-PAGE gel according to Sambrook (1989) using a Mini-Protean II apparatus (Bio Rad). Gels were blotted onto a BA85-S nitrocellulose membrane (Schleicher & Schuell) and membranes were incubated with antibodies as described (Sambrook *et al*., 1989). Rabbit anti AtSNAP33 serum was diluted 1:2000. Alkaline phosphatase conjugated secondary antibodies were diluted according to manufacturer's instructions (Dako). Reaction were visualized in

10 mM Tris buffer pH 9.6 containing 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg/ml of sodium 5-bromo-4-chloro-3-indolyl-phosphate (Eurobio) dissolved in dimethylformamide and 0.66mg/ml of 4-nitroblue tetrazolium chloride (Fluka) dissolved in 50% dimethylformamide.

Results

The expression of AtSNAP33 is increased by inoculation with *Plectosporium tabacinum*, *Peronospora parasitica* isolates NOCO and EMWA, and *Pseudomonas syringae* pv *tomato* carrying or not *avr* Rpt2

Figure 1 shows a time course of expression of AtSNAP33 and of PR1 after inoculation of *A. thaliana* accession Col-0 with the ascomycete *P. tabacinum*. PR1 encodes pathogenesis-related protein 1 which is secreted to the extracellular space and was taken as a marker of defense-related genes (Uknes *et al*., 1992).

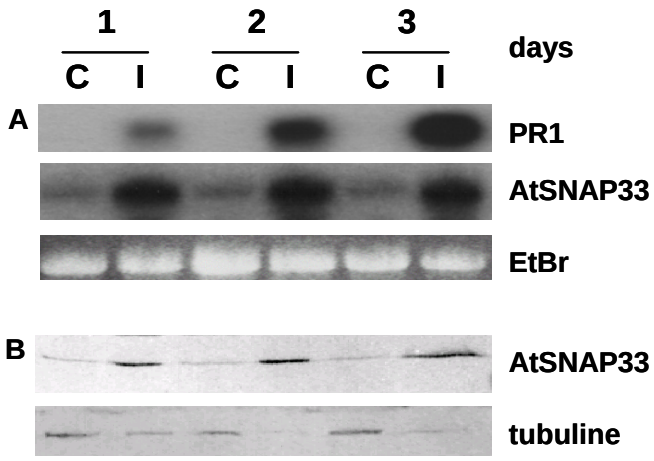

Figure 1: **Time course of expression of the *AtSNAP33* and *PR1* transcripts (A) and AtSNAP33 protein (B) after inoculation with *Plectosporium tabacinum*.** Leaves were inoculated with *P. tabacinum* (I) or water as a control (C) and collected after different times in days indicated above A. Six μg of total RNA were probed with the *AtSNAP33* and *PR1* cDNA probes. The RNA gel stained with ethidium bromide is shown as a control for loading. B, western blot of leaf proteins probed with anti AtSNAP33 antibodies. The western blot of leaf proteins probed with anti tubulin antibodies is shown as a control for loading.

The hyphae grew into the plant tissue and three days after inoculation, the leaves were yellow and the fungus sporulated. Four days after the inoculation, the leaves were completely macerated.

The level of *AtSNAP33* transcripts increased 1 day post inoculation (dpi) and remained high until 3 dpi compared to the level in control plants treated with water which showed very low expression of AtSNAP33. As a comparison, the level of *PR1* increased after 1 dpi and increased further 2 dpi and 3 dpi.

The expression of AtSNAP33 protein as visualized on immunoblots was induced 1 day after inoculation of the leaves with the pathogen, remained high until 3 dpi and was low in control leaves treated with water. The level of AtSNAP33 protein reflected the level of transcripts in Fig. 1 and many independent experiments (data not shown). Therefore, the level of *AtSNAP33* transcripts only was shown in further experiments.

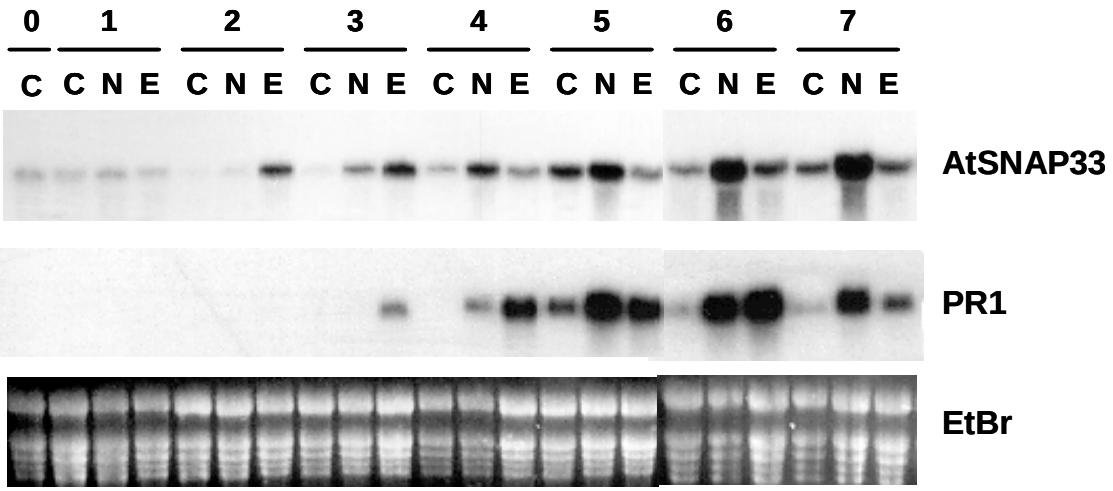

Figure 2: **Time course of expression of *AtSNAP33* and *PR1* transcripts after inoculation with *Peronospora parasitica* isolates NOCO and EMWA.** Leaves were inoculated with *P. parasitica* isolates NOCO (N) or EMWA (E) or water as control (C) and collected after different times in days as indicated. Six μg of total RNA were probed with the *AtSNAP33* and *PR1* cDNA probes. The RNA gel stained with ethidium bromide is shown as a control for loading.

Figure 2 shows the kinetic of expression of *AtSNAP33* and *PR1* after inoculation of *A. thaliana* Col-0 with the downy mildew pathogen *P. parasitica* isolate NOCO and EMWA. The isolate NOCO forms a compatible interaction and the isolate EMWA an incompatible interaction with *A. thaliana* accession Columbia (Parker *et al*., 1993, Holub *et al*, 1994). In the compatible interaction with the isolate NOCO, the hyphae grew into the plant tissues, forming numerous haustoria and no symptoms were

visible before 7 dpi when the conodiophores bearing the asexual conidia grew out of the stomata. During colonization, sexual spores formed as well. In the incompatible interaction with the isolate EMWA, the hyphae grew for 3 days forming haustoria. Then growth of the fungus stopped and trailing necroses of host cells along the length of the hyphae were observed. There was no asexual sporulation.

There was an increase of *AtSNAP33* transcripts 2 days and 3 days after inoculation with the isolate EMWA. The expression was back to control levels after 4 days. In contrast after inoculation with *P. parasitica* isolate NOCO, the level of *AtSNAP33* transcripts increased 4 dpi and remained high until 7 dpi. In the incompatible interaction with isolate EMWA, the level of *PR1* transcript increased after 3 days and stayed high until 7 dpi. In the compatible interaction with the isolate NOCO, the level of *PR1* transcripts increased 4 dpi and stayed high until 7 dpi.

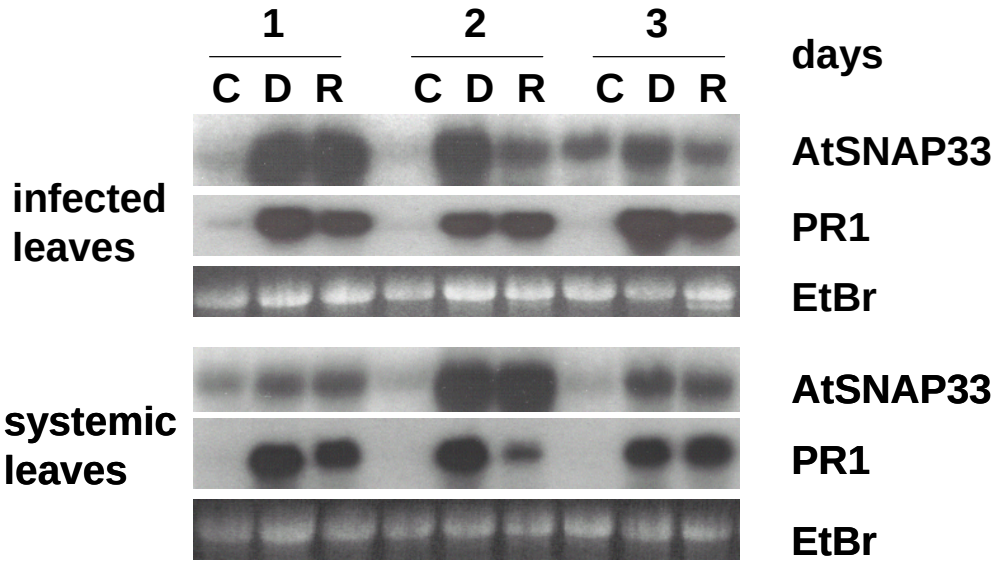

Figure 3: **Time course of expression of *AtSNAP33* and *PR1* transcripts in inoculated (A) and uninoculated systemic (B) leaves after inoculation with *Pseudomonas syringae* pv *tomato* DC3000 (D) or DC3000 *avr*Rpt2 (R) or water as a control (C).** Leaves were inoculated with the bacteria or water and inoculated as well as uninoculated sytemic leaves were collected after different times in days. Six μg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

Figure 3A shows a time course of the expression of *AtSNAP33* and *PR1* after inoculation with the bacteria *P. syringae* pv *tomato* DC 3000 carrying or not the

avirulence gene Rpt2. The expression of *AtSNAP33* increased 1 day after inoculation with the bacteria and stayed high for at least 3 days compared to controls which showed little expression of *AtSNAP33*. The expression of *PR1* was induced at the same time points, i.e. 1 to 3 dpi compared to controls which showed no expression of *PR1*.

Interestingly, the expression of AtSNAP33 was also induced in systemic non inoculated leaves of plants inoculated with *P. syringae* pv *tomato* DC3000 carrying or not *avr*Rpt2 (Figure 3B). As expected (Uknes *et al*., 1992), PR1 was also expressed systemically in inoculated plants.

The systemic expression of AtSNAP33 does depend on salicylic acid but not the expression in infected leaves

The dependence of the systemic expression of AtSNAP33 on the signalling molecule salicylic acid (SA) was tested in *nahG* transgenic plants which degrade SA to catechol (Gaffney *et al*., 1993). The plants were inoculated with *P. syringae* pv

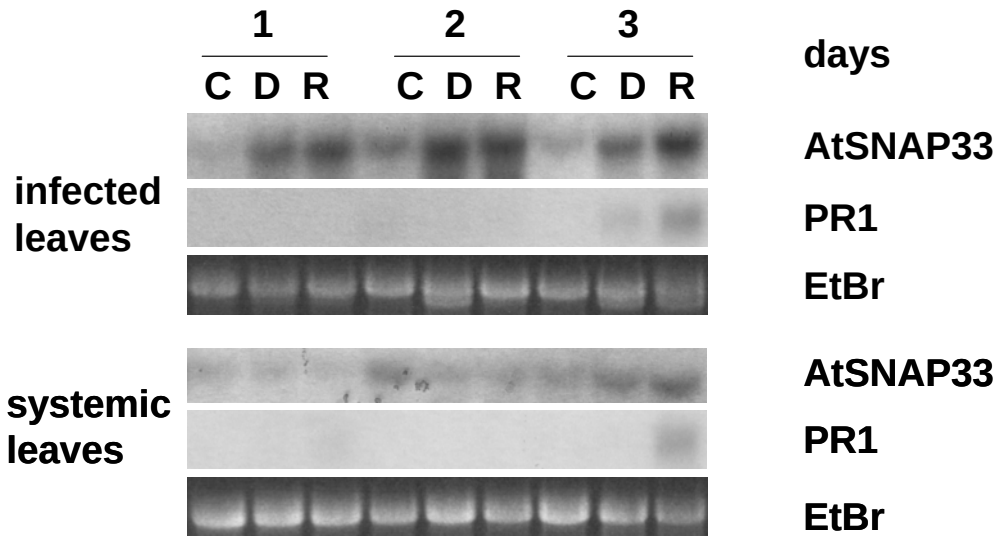

Figure 4: **Time course of expression of *AtSNAP33* and *PR1* transcripts in inoculated (A) and uninoculated systemic (B) leaves of *nahG* plants after inoculation with *Pseudomonas syringae* pv *tomato* DC3000 (D) or DC3000 *avr*Rpt2 (R) or water as a control (C).** Leaves were inoculated with the bacteria or water and inoculated as well as uninoculated sytemic leaves were collected after different times in days. Six μg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

tomato DC 3000 carrying or not avr Rpt2. Figure 4 shows that although *PR1* was not expressed in *nahG* plants either in inoculated leaves or in systemic uninoculated leaves, *AtSNAP33* was expressed in inoculated but not in systemic uninoculated leaves. Thus, the local induction of the expression of AtSNAP33 does not require SA in contrast to the systemic induction of expression which requires SA.

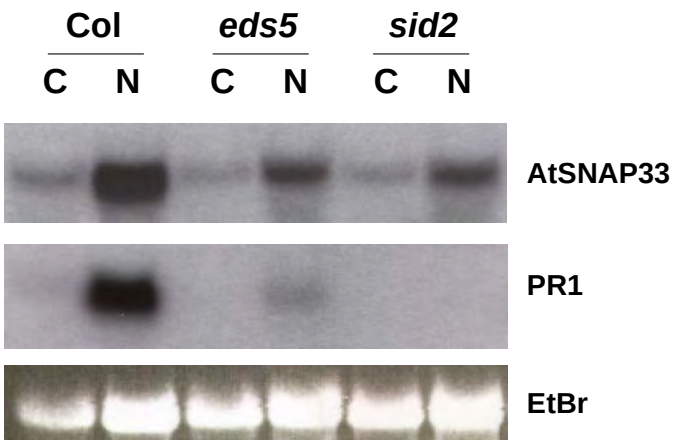

Figure 5: **Expression of *AtSNAP33* and *PR1* transcripts after inoculation of Columbia plants (Col), *eds5* and *sid2* mutants with *P. parasitica* isolate NOCO.** Leaves were inoculated with *P. parasitica* isolate NOCO (N) or water as a control (C) and collected after x days. Six μg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

Two other mutants unable to accumulate SA, the *eds5* and *sid2* mutants (Nawrath and Métraux, 1999) and wild type Arabidopsis Col-O were inoculated with *P. parasitica* isolate NOCO and the expression of *AtSNAP33* and *PR1* was analyzed (Figure 5). Six days after inoculation with the pathogen, *PR1* was not expressed in the *eds5* and *sid2* mutants as expected from previous reports (Nawrath and Métraux, 1999). However, *AtSNAP33* was still expressed but to a lower level than in wild type Col-O plants (Figure 5). The expression of *AtSNAP33* was very low in leaves of plants inoculated with water as a control.

As shown in *nahG* plants, expression of *AtSNAP33* after pathogen inoculation did not require SA in the inoculated leaves. Nevertheless, soil-drench application of 0.33 mM or 1 mM SA increased the level of expression of *AtSNAP33* in the absence of a pathogen as shown in Figure 6. This increase occurred already after 24 h but was higher after 48 h. When plants were treated by soil-drench application of 0.33 mM benzo(1,2,3)thiadiazole-7 carbonic acid S-methyl ester (BTH), an inducer of defense reactions, the expression of PR1 was induced as well as the

expression of *AtSNAP33* after 24 and 48 h.

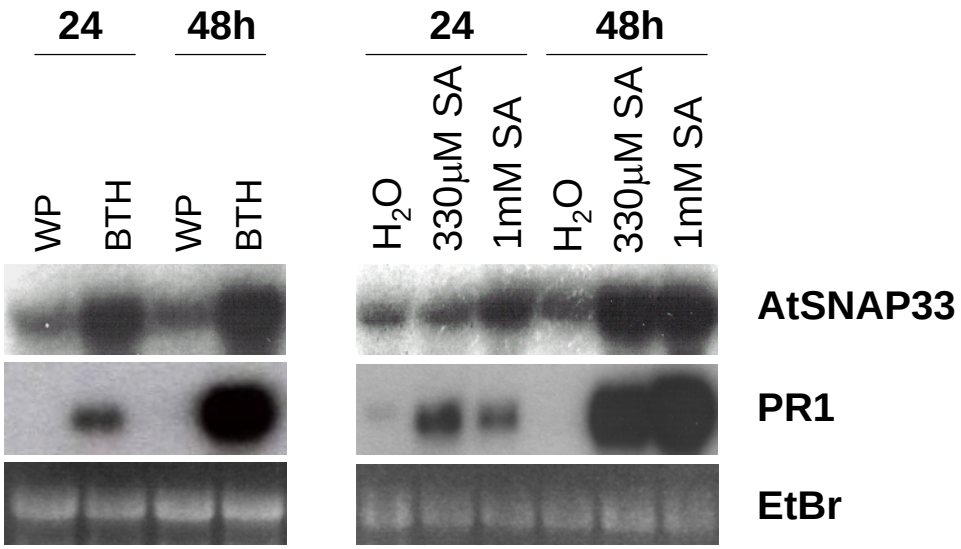

Figure 6: **Expression of *AtSNAP33* and *PR1* transcripts 24 h and 48 h after treatment with 0.33 mM BTH or 0.33 mM or 1 mM SA.** Wetting powder (WP) or water were used as a control for BTH or SA, respectively. Six µg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

In the *cpr1* mutant plants which overexpress PR1, PR2 and PR5 constitutively (Figure 7, Bowling *et al*., 1994), there was also a constitutive expression of *AtSNAP33* compared to wild type Col-O plants in the absence of a pathogen (Fig. 7). To see if *AtSNAP33* was expressed in conditions where PR1 is not expressed, the expression of *AtSNAP33* was analysed in the *npr1* mutant which shows no expression of PR1 and a reduced level of PR2 and PR5 after pathogen inoculation (Cao *et al*., 1994, Delaney *et al*., 1995). Figure 8 shows that there was no expression of *PR1* in *npr1* plants inoculated with *P. parasitica* isolate NOCO compared to wild type Col-O plants where *PR1* was expressed 4 dpi and the expression stayed high until 7 dpi. In the *npr1* mutants the expression of *AtSNAP33*

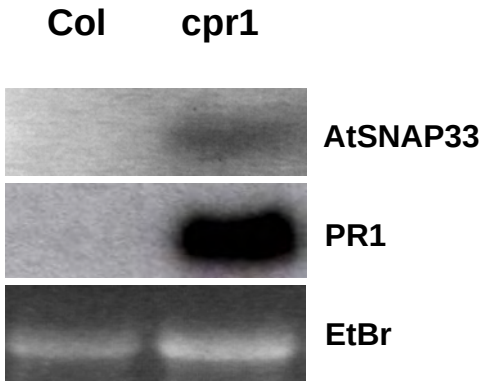

Figure 7: **Expression of *AtSNAP33* and *PR1* transcripts in leaves of Columbia plants (Col), and *cpr1* mutants.** Six µg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

increased 4 dpi and stayed high until 7 dpi similarly to the expression in wild type Col-O plants.

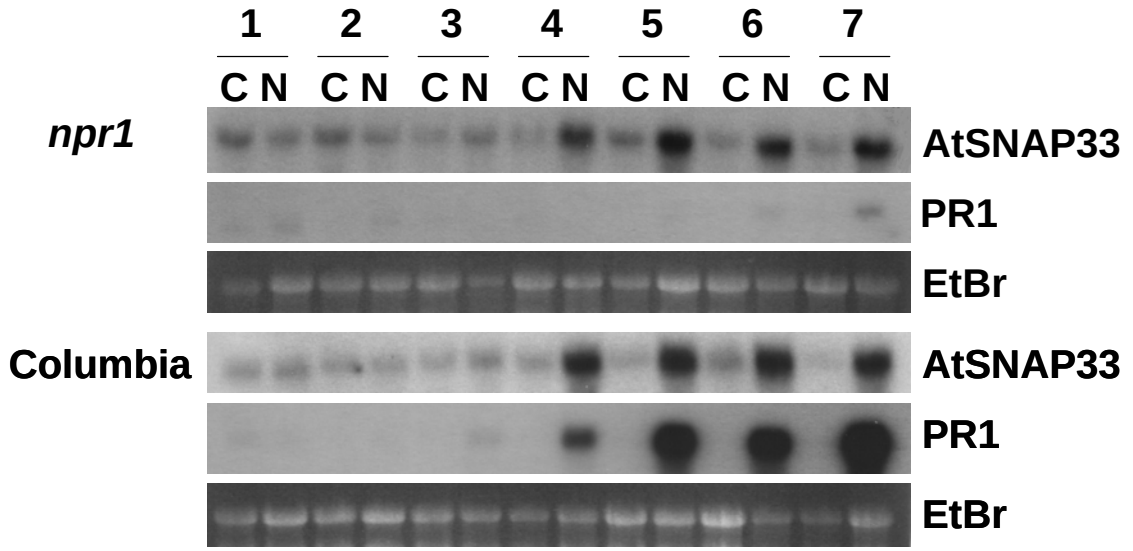

Figure 8: **Time course of the expression of *AtSNAP33* and *PR1* transcripts after inoculation of Columbia plants and *npr1* mutants with *P. parasitica* isolate NOCO.** Leaves were inoculated with *P. parasitica* isolate NOCO (N) or water as a control (C) and collected after different times in days as indicated. Six μg of total RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

The expression of AtSNAP33 is induced after mechanical stimulation and wounding

Figure 9 shows the expression of *AtSNAP33* after touching of the leaves compared to control untouched leaves. The level of *AtSNAP33* transcripts increased transiently 15 minutes after a mechanical stimulation which consisted in rubbing gently the leaves 10 times between the thumb and forefinger, reached a maximum around 30 minutes and

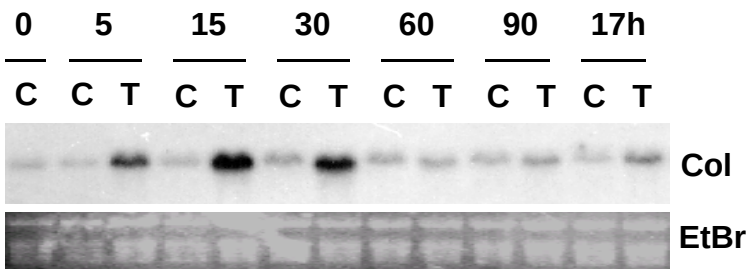

Figure 9: **Time course of expression of *AtSNAP33* transcripts after mechanical stimulation.** Leaves were touched (T) or not touched as a control (C) and collected at time 0 and after 5, 15, 30, 60, 90 min and 17 h. Six μg of total RNA were probed with the *AtSNAP33* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

decreased to control levels after 90 minutes. To test if the enhanced expression was due to breakage of trichomes which occurs by touching the leaves, the expression of *AtSNAP33* was analysed after mechanical stimulation in *A. thaliana glabrous1* mutant plants which bear almost no trichomes (Koornneef *et al.*, 1982).

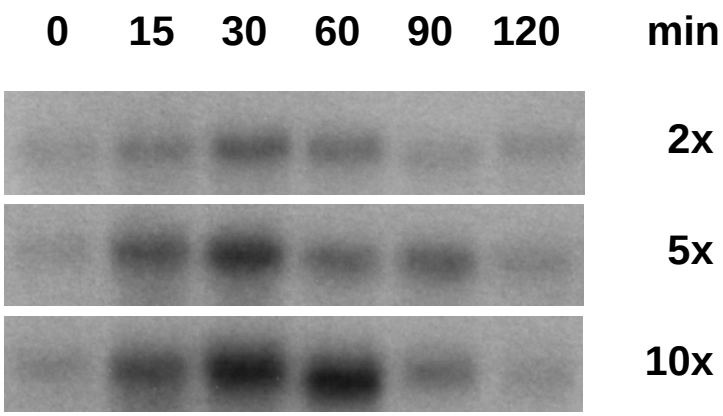

Figure 10: **Time course of expression of *AtSNAP33* transcripts after different intensities of touch.** Leaves were touched twice (2x), five times (5x) or 10 times (10x) and collected after different times in min as indicated. Six μg of RNA were probed with the *AtSNAP33* cDNA probe.

Like in wild type plants, the expression of *AtSNAP33* in the mutant followed a similar transient increase. The expression of *AtSNAP33* was not increased in upper systemic leaves after touching the lower leaves (data not shown).

The level of the expression of AtSNAP33 depended on the intensity of the touch treatment. This expression was already increased when the leaves were touched twice but was higher when the leaves were touched five or ten times (Figure 10). The kinetics of expression were similar with different intensities of touching.

The effect of two other mechanical stimulation, i.e. wind and wounding, on the

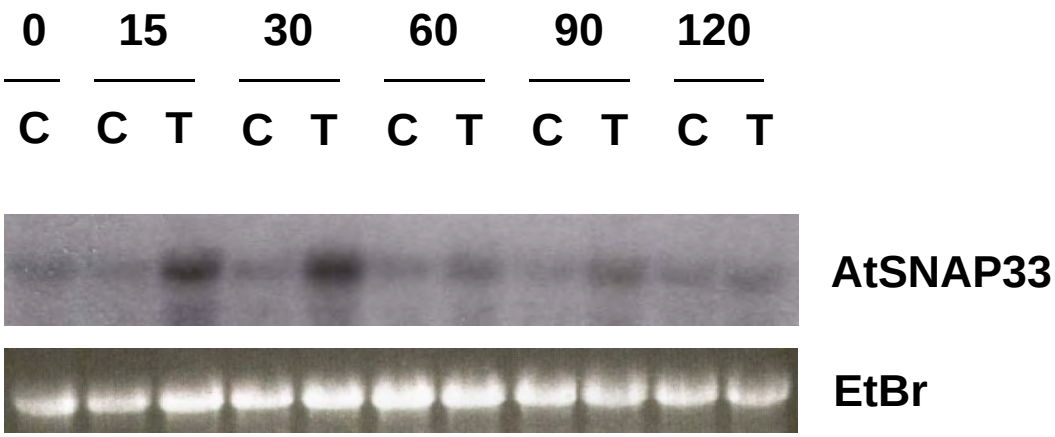

Figure 11: **Time course of expression of *AtSNAP33* transcripts after exposure to wind.** Leaves were exposed to a hair drier (T) or not exposed as a control (C) and collected after different times in min as indicated. Six μg of RNA were probed with the *AtSNAP33* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

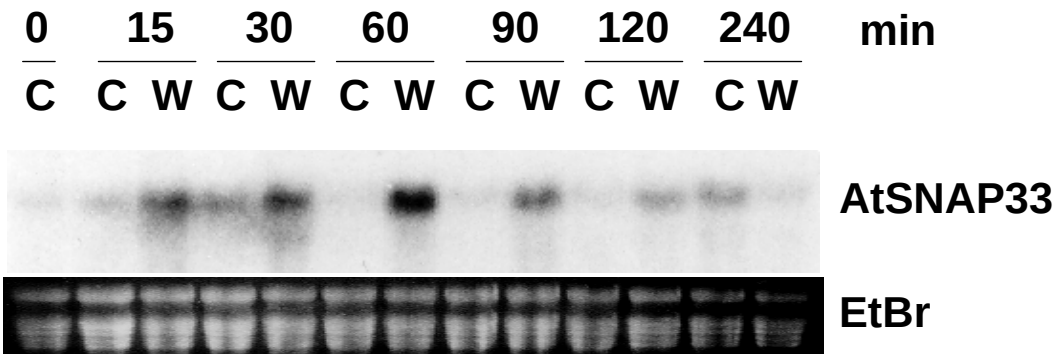

Figure 12: **Time course of expression of *AtSNAP33* transcripts after wounding.** Leaves were wounded (W) or not wounded as a control (C) and collected after different times in min as indicated. Six μg of RNA were probed with the *AtSNAP33* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

expression of AtSNAP33 was tested. Wind, provided by an air drier similarly caused a transient increase of the expression of AtSNAP33 after 15 minutes and 30 minutes compared to control untreated plants and the expression was back to control levels after 120 minutes (Figure 11). The level of AtSNAP33 also increased transiently after wounding. It increased after 15 min, reaching a maximum after 60 min and returning to levels of unwounded leaves 240 min after beginning of the experiment (Figure 12).

AtSNAP33 and PR1 are expressed after UV irradiation

The induction of AtSNAP33 was also investigated in conditions that were free of mechanical interference. For instance, a strong induction was observed after UV treatment. Plants were treated for 20 minutes with UV-C irradiation which induce the expression of PR1 (Yalpani et al., 1994).

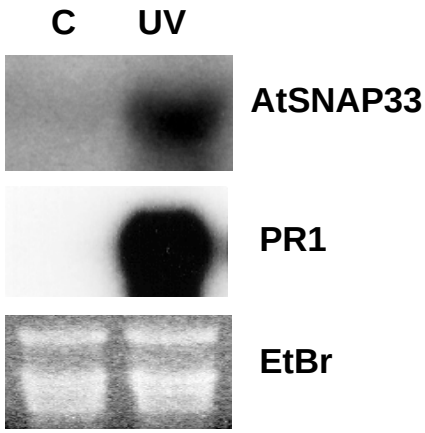

Figure 13: **Expression of *AtSNAP33* and *PR1* transcripts after UV irradiation.** Plants were exposed to UV light for 20 min (UV) or to day light as a control (C) and leaves were collected after 17 h. Six μg of RNA were probed with the *AtSNAP33* or *PR1* cDNA probe. The RNA gel stained with ethidium bromide is shown as a control for loading.

Samples were collected 17 h after the irradiation and the expression of *AtSNAP33* and *PR1* was analysed (Figure 13). The level of *AtSNAP33* transcripts increased in treated plants compared to control untreated plants. The level of *PR1* transcripts were also increased after UV irradiation compared to controls.

Discussion

We show herewith that the expression of the t-SNARE AtSNAP33 increased after pathogen inoculation, mechanical stimulation and UV irradiation. In the experimants involving treatment with a pathogen, the expression of PR1 was taken for comparison as a marker for PR protein expression. The expression of AtSNAP33 was low in untreated leaves, in agreement with the low secretory activity of these leaves.

The expression of AtSNAP33 increased after inoculation with the pathogen *P. parasitica* isolates NOCO and EMWA. There was a faster increase in the expression of AtSNAP33 in the incompatible interaction with isolate EMWA than in the compatible interaction with isolate NOCO.

The expression of AtSNAP33 correlated with the colonization of the leaf by *P. parasitica.* Indeed, in the incompatible interaction with isolate EMWA, the hyphae grew into the tissue and formed haustoria until 3 dpi and then growth stopped. In this case, the expression of AtSNAP33 increased 2 and 3 dpi and then was back to control levels. In the compatible interaction with isolate NOCO, the colonization of the tissue continued and the expression of AtSNAP33 increased 2dpi and increased further until 7 dpi. Haustoria were continously formed by the growing hyphae. These specialized structures grow into plant cells without rupturing the plasma membrane and are surrounded by an invagination of a modified form of the host plasma membrane, the extrahaustorial matrix. The growth of the plasma membrane to surround the haustoria in formation is thought to occur by fusion of vesicles, the

membrane of the vesicle becomig part of the extrahaustorial membrane (Callow and Green, 1996) and may require an increased level of components of the vesicle fusion machinery such as AtSNAP33.

Similarly, an enhanced level of the t-SNARE AtSNAP33 can be expected when secretion of proteins is increased leading probably to more vesicle fusion events with the plasma membrane. Indeed, analysis by western blots of intercellular wash fluids showed that there was an increased secretion of PR1 after inoculation with *P.parasitica* isolates NOCO and EMWA and *P. tabacinum* (data not shown).

In the compatible interaction with isolate NOCO, the expression of *PR1* increased 4 dpi and increased further until 6 dpi. It is interesting to note that the increase in the expression of *AtSNAP33* preceeded the increase in the expression of *PR1* indicating that it does not result from a feedback mechanism whereby an increased secretion would lead to an increased expression of *AtSNAP33*. Moreover, by 4 to 6 dpi with isolate EMWA, the expression of *AtSNAP33* returned to control levels when the expression of *PR1* was still high. These differences in the expression of *AtSNAP33* and *PR1* suggest that the expression of the 2 genes are regulated by different signalling pathways.

The expression of AtSNAP33 was induced in the *nahG* plants, and *eds5* and *sid2* mutants after inoculation with *P. parasitica* isolates NOCO and EMWA. These plants are unable to accumulate SA after pathogen inoculation and show enhanced susceptibility to both virulent and avirulent strains of *P. parasitica* (Delaney *et al*., 1994; Nawrath and Métraux, 1999). The avirulent strain can complete its life cycle in the mutants. There is a low level of expression of *PR1* in the *nahG* plants inoculated by pathogens (Delaney *et al*., 1994, Fig.4). In *eds5* and *sid2* plants, *PR1* is expressed only to a low level but *PR2* and *PR5* are still expressed as in wild type plants after pathogen inoculation (Nawrath and Métraux, 1999). The increase of AtSNAP33 in the *nahG* plants, and *eds5* and *sid2* mutants after inoculation with *P. parasitica* indicate that the regulation of its expression is not mediated by the SA-dependent signalling pathway in contrast to the expression of *PR1*. It may be linked

to the secretion of *PR2* and *PR5* in the *eds5* and *sid2* mutants and to the enhanced growth of the pathogen forming numerous haustoria.

The expression of AtSNAP33 also increased after inoculation with 2 other pathogens, *P. tabacinum* and *P. syringae* pv *tomato* carrying or not *avr*Rpt2. Thus, the expression of AtSNAP33 increased after treatment with 3 different pathogens and may be related to the increased secretion of PR proteins and in the case of *P. parasitica* also to the formation of the extrahaustorial membrane. We postulate that an increased secretory activity could be correlated to a higher level of components of the exocytotic fusion machinery as indicated by the upregulation of AtSNAP33 after pathogen attack. However, the involvement of AtSNAP33 in secretion remains to be shown. Expression of *AtSNAP33* in the *cpr1* mutant could be the consequence of the constitutive expression of PR1, PR2 and PR5 or to enhanced level of SA. Indeed, SA was sufficient but not necessary to induce the expression of AtSNAP33. The signalling pathway leading to the expression of AtSNAP33 after pathogen inoculation did not depend on SA in inoculated leaves as AtSNAP33 was expressed in inoculated leaves in *nahG* plants in contrast to PR1 which was not expressed. However, systemic expression of AtSNAP33 required SA as it was abolished in *nahG* plants.

It was found serendipitously that the expression of *AtSNAP33* increased 15 min after mechanical stimulation, reaching a maximum after 30 to 60 min. Ninety min after mechanical stimulation, the expression was again at the level of expression in control untreated leaves, indicating a rapid turnover of the transcripts. The expression of *AtSNAP33* show the same characteristics as the expression of other genes induced by mechanical stimulation including the *TCH* genes (Braam *et al*., 1997), i.e. the increase in the level of transcript was high, rapid and transient. When the leaves were mechanically stimulated once every day for 35 days, there was a decrease in the height of the inflorescence stem (26% of control, untouched plants). Thus, mechanical stimulation lead to changes in development which may be related to cell wall modifications. The increased expression of *AtSNAP33* after mechanical

stimulation may reflect an increase in secretion of cell wall components. Indeed, *TCH4* which is induced after mechanical stimulation encodes a xyloglucan endotransglycosylase and possibly functions in cell wall modifications following mechanical stimulation leading to changes in development (Xu *et al*., 1995, Braam *et al*., 1997).

The increase in the expression of AtSNAP33 after touch stimulation was not caused by breakage of trichomes since the expression of *AtSNAP33* also increased after mechanical stimulation of mutant *glabrous1* plants which bear almost no trichomes (Koorrneef *et al*., 1982).

The expression of *AtSNAP33* increased transiently after wounding similarly to the increase after mechanical stimulation. Since the expression of *AtSNAP33* was back to control levels 240 min after wounding, it may be a consequence of the mechanical stimulation occurring during wounding rather than a response to the damage provoked by the wound.

As AtSNAP33 is a t-SNARE localized at the phragmoplast and at the plasma membrane (Heese *et al*., 2001), we hypothesize that it is involved in vesicle fusion for the formation of the cell plate after cell division and for secretion of extracellular components. After pathogen attack increased vesicle fusion may be required to permit increased secretion of PR proteins. In addition, increased vesicle fusion may also be required for repair of damage to the plasma membrane provoked by reactive oxygen species. Reactive oxygen species are produced after pathogen attack, UV treatment and mechanical stimulation (Yahraus *et al*., 1995). It has been shown in see urchin eggs that AtSNAP-25 is required for membrane resealing after injury (Steinhardt *et al*., 1994). Moreover, overexpression of AtVAMP7, an Arabidopsis v-SNARE prevented H_2O_2-induced apoptosis in yeast and Arabidopsis cells (Levine *et al*., 2001).

To our knowledge, this is the first report of the induction of the expression of a SNARE after pathogen inoculation, mechanical stimulation and UV irradiation. It will be interesting to analyse the expression of other SNAREs in response to these

stimuli.

Acknowledgments

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The „knockout“ of the t-SNARE AtSNAP33 in *Arabidopsis* provokes a lesion mimic phenotype and a reduced secretion activity

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Key-words: *Arabidopsis*, secretion, SNAP-25, t-SNARE, lesion mimic

Abstract

The fusion of vesicles in the secretory pathway involves the interaction of membrane associated proteins, soluble N-ethylmaleimide sensitive proteins receptors or SNAREs on the target membrane (t-SNAREs) and on the vesicle membrane (v-SNAREs). AtSNAP33 is a homologue of the neuronal t-SNARE involved in exocytosis SNAP-25 and is located at the plasma membrane. AtSNAP33 is interacting with KNOLLE, an Arabidopsis syntaxin (t-SNARE) which is involved in the formation of the new cell plate by Golgi-derived vesicles during cytokinesis and NtSYR1 a syntaxin homologue of tobacco which is involved in the signalling pathway of ABA in guard cells. The „knockout“ mutant *atsnap33* has a lethal, dwarf phenotype. Seven days after germination the formation of reactive oxygen intermediates (ROIs) and lesion formation were observed on the cotyledons. The lesions spread out over the whole plant except the roots and after 3 - 4 weeks the upper green part of the plant was dead. In 7 day old *atsnap33* mutants the defence genes PR-1, PR-2, and PDF1.2 were upregulated whereas the two homologues of AtSNAP33, AtSNAP30 and AtSNAP29 were not expressed. The root growth rate of the mutants was lower compared to the wild type grown in the same conditions. *Atsnap33* mutants and wild type plants were transformed with a secreted barley α-amylase under the control of the 35S promoter for constitutive expression. During the first 7 days there was no differences in secretion of α-amylase between wild type Columbia and the mutant. But after 7 days the secretion of the α-amylase was inhibited in the *atsnap33* mutant. This correlates exactly with the appearance of the lesion mimic phenotype. The reduction of the root growth together with inhibited secretion of α-amylase indicate that AtSNAP33 is necessary for secretion.

Introduction

The plant secretory pathway consists of different membrane bounded organelles including the endoplasmic reticulum (ER), the Golgi apparatus and the vacuole. Membrane-bound vesicle mediate transport between the compartments of the secretory pathway and the plasma membrane. Secreted proteins have a signal peptide and are synthesized at the ER. The signal peptide targets the protein in the lumen of the ER. The polypeptides are folded in the lumen and packaged into vesicles that bud from the ER and move to the Golgi where they fuse. The proteins are transported through the Golgi and at the trans-Golgi network (TGN) secreted proteins are sorted from vacuolar proteins. Secreted proteins are transported by vesicles to the plasma membrane. Exocytosis, the fusion of the vesicles with the plasma membrane and the release of their content to the extracellular space is the last step of the secretory pathway.

Secretion is involved in different processes in plant development and cell growth including cell wall formation, pollen tube growth, xylem differentiation, slime formation by the root cap and the formation of the cell plate by fusion of Golgi-derived vesicles during cytokinesis (for reviews see Battey and Blackbourn, 1993; Thiel and Battey, 1998). The tip growth of single cells like pollen tubes is dependent on a high secretory activity. Vesicles fuse at the tip at rates of up to 6000 vesicles per minute in pollen tubes (Thiel and Battey, 1998). Molecules transported through the plant secretory pathway include proteins, lipids, polysaccharides and cell wall components except cellulose and callose.

The maintenance of the cellular compartmentalization is essential for the function of the cell and requires correct transport between the compartments allowing fusion of vesicles only with the appropriate membrane.

Söllner *et al*., (1993) characterized membrane associated proteins called SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) which are located on vesicles as well as on the plasma membrane and are necessary for vesicle fusion in neurons. All SNAREs have a 60 amino acid peptide stretch called the SNARE motif (Fasshauer *et al*., 1998), which is able to interact with the corresponding SNARE partners. Sequencing of different genomes like human being, yeast and *Arabidopsis* revealed a high number of SNAREs which are distributed among the organelles involved in the secretory pathway. Crystal analysis of the

neuronal SNARE complex showed that the SNARE motifs of SNAREs form a twisted four-helix bundle SNARE core complex (Weber *et al*., 1998; Sutton *et al*., 1998). The assembled complex consists of one SNARE localized on the vesicle membrane (v-SNARE) containing one SNARE motif and two SNAREs localized on the target membrane (t-SNARE). For t-SNAREs on the target membrane, the syntaxin-like protein supplies one and a SNAP-25-like protein contributes two SNARE motif (Fasshauer *et al*., 1998).

Sequencing of the genome of *Arabidopsis* uncovered a remarkable number of SNARE proteins but the precise function of most of them is not known. Twenty-four syntaxins were found by homology and classified in 8 classes by Sanderfoot *et al*., (2000) but only few syntaxins are functionally characterized yet. *Arabidopsis* possess three SNAP-25 like proteins and over 20 expressed synaptobrevin-like proteins (v-SNAREs).

Recently the *Arabidopsis* t-SNARE AtSNAP33, a SNAP-25-like protein, was described to interact with the syntaxins from *Arabidopsis* KNOLLE (Heese *et al*., 2001) and from tobacco NtSYR1 (Kargul *et al*., 2001). AtSNAP33 is located at the plasma membrane and behaves as an integral membrane protein (Heese *et al*., 2001). A T-DNA insertion in the AtSNAP33 gene caused loss of AtSNAP33 function, resulting in a lethal dwarf phenotype. *Atsnap33* plantlets develop large necrotic lesions on the cotyledons and rosette leaves similar to the lesion mimic phenotype (Heese *et al*., 2001). Lesion mimic mutants have been identified in plants and spontaneously form localized areas of dead tissue resembling those seen in the hypersensitive reaction (HR) but in the absence of pathogens. HR is a possible defence reaction of the plant after pathogen attack. The host cell death is manifested as a rapid collapse of cells around the site of penetration of the pathogen (for a review see Sticher *et al*., 1997) The reason for lesion formation in these mutants can be either the mutation of genes for which the wild type functions are involved in disease resistance or the mutant cell death phenotype results from perturbed metabolism that interferes with the process of programmed cell death (PCD) (for review see Mittler and Rizhsky, 2000). A marker for PCD is the production of reactive oxygen intermediates (ROIs). Superoxides formed by NAD(P)H oxidases and / or peroxidases are enzymatically converted in hydrogen peroxide and other more noxious ROIs. The accumulation of ROIs can cause directly cell death or act as a

signal and trigger an independent cell death pathway (for reviews see Heath, 2000; Shirasu and Schulze-Lefert, 2000). Antioxidants such as diphenyleneiodonium (DPI) reduce in a dose-dependent manner lesion formation in the *lsd1* mutant of Arabidopsis by inhibition of the plasma membrane NAD(P)H oxidase. (Jabs *et al*., 1996).

In this report we characterize in a first part the „knockout“ mutant of AtSNAP33, a SNAP-25 like protein in Arabidopsis and in particular its lesion mimic phenotype . In the second part of this work we found that the secretory activity in *atsnap33* roots was inhibited, taking a barley α-amylase as a secreted marker.

MATERIALS AND METHODS

Plant material

A. thaliana accession *Columbia* (Col)

A. thaliana ecotype Wassilewskija (WS)

eds5 / *sid2* / *cpr1* / *npr1*/ NahG / *glabrous1* (ecotype Col)

Atsnap33 EFS396 (T-DNA insertion line originated from the Versailles T-DNA collection (ecotype WS))

Arabidopsis transformed with the α-amylase cDNA pM/C (ecotype Col)

Arabidopsis transformed with the α-amylase cDNA Clone E (ecotype Col)

Kits and Enzymes

For subcloning of fragments obtained by the polymerase chain reaction (PCR) the pGEM®-T easy vector system (Promega) was used. High quality plasmid DNA preparation were done using Machery & Nagel Midi Kit; Qiagen Mini Prep; Promega

Midi Prep Wizard Plus kit. For the purification of DNA fragments from agarose gels the Qiagen gel extraction kit and Machery & Nagel Nukleobond kit were used.
All restriction endonucleases were provided by Gibco BRL and New England Biolabs and used as described in manufacturers instruction.
General molecular biology techniques were performed according to Sambrook et *al.*, (1989) standard protocols.

Bacterial Strains

For cloning, *E.coli* XL1-blue (resistant to 10 μg/ml tetracycline), DH5α and DH10B were used. *Agrobacterium tumefaciens* strain GV3101 (pMP90 helper plasmid, resistant to 25 μg/ml gentamycin, resistant to 100 μg/ml rifampicin) was used for plant transformation.

Bacterial growth conditions

The different strains of *E. coli* were grown at 37°C in LB liquid medium (Miller's Luria broth base, Sigma) with continuous shaking or in Petri plates on Luria Broth with 1% agar. For the selection of the transformed bacteria the adequate antibiotics were used.

Plant growth conditions and seed sterilisation

Arabidopsis plants sowed on soil and startified in the dark at 4°C for 4 - 5 days were grown in growth chambers under short day conditions (12 h light / 12 h dark) at 20°C. For *in vitro* culture the seeds were washed briefly with 70% ethanol and sterilised for 20 min with 2.5 % bleach containing 0.05 % Triton X100. The bleach was removed with 5 washes with sterile water then the seeds were placed on ½ MS (Murashige

and Skoog) with 1% sucrose and 0.7% agar. After startification the plants were grown in the same conditions as in soil unless otherwise stated.

The „knockout“ mutant *atsnap33* showed in a lethal dwarf phenotype. Seven days after germination the mutants develop formation of spontaneous and spreading necrosis. After three to four weeks the plantlets are dead. *Atsnap33* is not fertile and has to be maintained in the heterozygote stage. The seeds of heterozygote plants which were first tested by PCR according to the protocol of Heese et *al.*, (2001) were grown on a selective ½ MS medium containing 50 μg / ml kanamycin. Only 50% of the population of the seedlings the heterozygotes containing the resistance to kanamycin survived. 25% of the population the wild type were kanamycin sensitive and will die and the 25% correspond to the homozygotes which died after 3 – 4 week. The mutant *atsnap33* were selected and analysed 7 days after germination when the formation of the necrosis were well established compared to the wild type WS plants.

For the root growth measurement seeds from *atsnap33* mutants and wild type were sterilised as described above. The seeds were placed in a line on Petri plates and after stratification of 4 - 5 day at 4°C, the plates were put vertically in the growth chamber (12h light / 12 h dark) at 20°C. The length of the roots were measured daily.

Antioxidant application

For experiments using antioxidants 3 day young plantlets grown *in vitro* on ½ MS medium were treated with a droplet of 50mM or 500mM ascorbic acid (Fluka), 5mM or 50mM DTT (dithiothreitol, Fluka), 1mM or 10mM KI or 0.5mM or 5mM BHT (buthylated hydroxytoluene, Fluka). Then the plates were placed in the growth chamber (Bethke et *al.*, 2001). Survival of the plantlets was monitored.

Floral dip transformation of *Arabidopsis* plants with *Agrobacterium tumefaciens*

For *Arabidopsis* transformation, the floral dip method (Clough and Bent, 1998) was used with slight modifications. *Agrobacterium tumefaciens* strain GV3101 was transformed by electroporation with the construct designed for plant transformation. Single colonies were used to inoculate a pre-culture in 5 ml of LB medium (containing 25 μg/ml gentamycin, 100 μg/ml rifampicin and the corresponding antibiotic used for construct selection) and grown overnight at 28°C on a shaker at 220 rpm. Five ml of the pre-culture were used to inoculate 500 ml LB medium (containing 25 μg/ml gentamycin, 100 μg/ml rifampicin and the corresponding antibiotic used for construct selection) and grown overnight at 28°C on a shaker at 220 rpm. The bacteria from a 500 ml culture were harvested by a 10 min centrifugation at 5000 g at RT and resuspended in the infiltration medium (5% sucrose, 0.05% Silwett L-77) to give an OD_{600} of 0.6 was reached. Withered flowers and siliques were cut off from 4 to 6 week old plants so that only flower buds or lightly opened flowers were left over. The inflorescences of plants were dipped for about 30 seconds into the infiltration medium. After dipping, the plants were laid horizontally into a tray and the tray was covered overnight with a plastic bag. When the plastic bag was removed, the plants were put upright and cultured as normal.

Selection of transgenic plants

All transgenic plants generated in the course of this work were co-transformed with a kanamycin or hygromycin B selectable marker. For selection of transformants T1-seeds were sterilized and placed on selection plates (1/2 MS, 1% sucrose, pH 5.8, 50 μg/ml kanamycin or 35 μg/ml hygromycin B). Resistant plants developed into green seedlings and were transferred to soil after the first true leaves appeared.

Lactophenol - Trypan blue staining

For trypan blue staining, leaves or whole plantlets were covered with an alcoholic solution of lactophenol trypan blue: lactophenol trypan blue : 95% ethanol 1 : 2 (vol :

vol). The staining solution with the samples was boiled in a water bath for 1 min. Then the plant tissues were incubated overnight in the staining solution and discoloured with 4 g / ml chloral hydrate until totally transparency as described by Keogh et *al.*, (1980). The stained leaves were further analysed by microscopy.

Active oxygen species detection

In situ production of 0_2^- in the „knockout“ mutant *atsnap33* was detected with NBT (nitroblue tetrazolium 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3-3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride, Fluka) that forms a dark blue insoluble precipitate in the presence of 0_2^-. The method described by May et *al.*, (1996) was used with the following modifications: 7 day-old plantlets were collected and infiltrated immediately with 0.5% (w/v) NBT dissolved in 10mM K_2HPO_4 pH 6.0. Infiltrated plantlets were incubated at RT for 15 min. Then incubated over night in Chloral hydrate 4 g / ml which stops the reaction and bleaches the leaves. The stained leaves were further analysed by microscopy.

In situ production of H_2O_2 in 7 day-old plantlets was detected by the formation of a brownish precipitate in the presence of endogenous peroxidase after infiltration of DAB (diaminobenzidine) according to Thordal-Christensen et *al.*, (1997). DAB (2 mg/ml) was dissolved in water and freshly made. The whole plantlets were vacuum-infiltrated with DAB and incubated at RT overnight. The reaction was stopped and leaves were bleached with 4 mg/ml chloral hydrate. The stained leaves were further analysed by microscopy.

Microscopy

Different tissues were observed with the microscope DM R (Leica). The pictures were taken with the CCD Camera AxionCam (Zeiss), analysed and prepared with the software Axion Vision 2.05. Images were processed with Adobe Photoshop® 5.0.

Isolation of genomic DNA from *Arabidopsis thaliana*

Leaves of *A. thaliana* were ground in liquid nitrogen. To each gram of plant material 10 ml of EB-buffer (100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 1,4% SDS, 0.001% β-mercaptoethanol) preheated to 70°C, were added. After 10 minutes incubation at 70°C 4 ml of 5 M sodium acetate were added and the solution was mixed. After centrifugation for 20 min at 10'000g 0.6 volumes of isopropanol were added and the solution was centrifuged for 10 min at 10'000g. The dried pellet was resuspended in 700 µl TE-buffer (10 mM Tris pH 8, 1 mM EDTA pH 8), treated with RNase A (final concentration 50 µg/ml) for 10 min at 37°C. One volume of phenol/chloroform was added and the solution was centrifuged for 10 min at 14'000 rpm in an Eppendorf 5415C centrifuge. The supernatant was washed once with 1 volume of chloroform and centrifuged. The genomic DNA was precipitated in 0.7 volumes of isopropanol and washed in 70% ethanol. The pellet was resuspended in 200 µl of TE-buffer.

RNA preparation, RNA blot analysis and probes of *AtSNAP33*, *AtSNAP29* and *AtSNAP30*

For RNA extraction, plant tissues were harvested, frozen and pulverized in liquid nitrogen. One volume of 2 M Tris pH 8 containing 0.5 M EDTA pH 8 and 20% SDS (v:v:v; 1:2:1) at 95°C was added, followed by 1 volume of saturated phenol:chloroform:isoamyl alcohol (v:v:v; 25:24:1) at 40°C. The two phases were separated by centrifugation, the aqueous phase containing the RNA was mixed with one volume of chloroform and separated by centrifugation. The RNA was precipitated with 1 volume of 6 M LiCl for at least 2 h or overnight at 4°C, washed with 70% EtOH and resuspended in water. For RNA blot analysis, 6 µg of total RNA were applied on a 1% formaldehyde-agarose gel, separated and transferred to a nylon membrane (Hybond-N Amersham) as described by Sambrook et *al.*, (1989). The membranes were hybridised overnight at 65°C with a DNA probe made by random primed labelling in the presence of α-32P dCTP with the RadPrimed DNA Labeling System according to manufacturer's instructions (Life Technologies or Promega). The

membranes hybridised with the described probes, were washed subsequently for 5 min with 2 x SSC (0.3 M NaCl, 0.03 M Trisodium citrate) containing 0.1% SDS and then 3 times for 20 min with 0.2 x SSC containing 0.1% SDS at 65°C before exposing to X-Omat film (Kodak).

For probes for Northern blot analysis *AtSNAP33*, *AtSNAP30* and *AtSNAP29* the PCR technique was used. The set of primers 5'GAG TCG TCT CCG CGT TA3' and 5'ATC ACG GAA ATT GTT CTT G3' were used to amplify 500 bp of the non conserved part of the *AtSNAP33* cDNA by PCR using the Taq - Polymerase of Sigma and cloned in the pGEM®-T easy (Promega) according to the manufacturer's instruction. The specific probe for *AtSNAP29* and *AtSNAP30* were amplified from genomic DNA from *A. thaliana* accession *Columbia* plants by PCR with the set of primers 5'CTT CCA AGT CGA TTT AGG TCT AC3' and 5'CGA GGG CTT CAA GGA ATG AAG TG3' for *AtSNAP29* and the set of primers 5'TTA ATA TGG GTC TTG ACA TTT CTC C3' and 5'GTT GAA ACC GTT CTT GTA TTT GTC G3' for *AtSNAP30*. The 434 bp fragment of *AtSNAP29* and the 477 bp fragment of *AtSNAP30* were cloned in the T-cloning sites of pGEM®-T easy vector (Figure 2). All three probes are in the 5' region of the cDNA.

Arabidopsis transformation with two different α-amylases from barley

The two cDNAs from different α-amylases pM/C and Clone E were isolated from barley and were a kind gift of J.C. Rogers (Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA). The cDNAs were transferred in the binary vector pBin19 containing a double 35S cauliflower mosaic virus promoter. This work was performed by I. Studer and V. Page during their diploma thesis. *Arabidopsis thaliana* ecotype Columbia were transformed by the floral tip method described above.

AlcA promoter::*AtSNAP33*p constructs

The plasmid pACN*SNAP33p* was constructed using the pACN plasmid (Caddick et *al.*, 1998), where the chloramphenicol acetyltransferase (CAT) gene was replaced by a part of the cDNA of *AtSNAP33*. The set of primers 5'TTT CTG CAG TTG GTT TGA TTG TTG CAC3' and 5'AAA CTG CAG ATG TTT GGT TTA AGG AAA TC3' both containing a *Pst*I site was used to amplify *AtSNAP33* without the 10 amino acids at the C-terminus (*SNAP33p*) from the cDNA of *AtSNAP33*. *SNAP33p* (aa 1 – 290) was sequenced and cloned in the *Pst*I site of pACN replacing the CAT gene. The reporter cassette AlcA::*SNAP33p* was cloned in the *Hin*dIII site of the binSRNACatN plasmid (without AlcA::CAT) as is recommended by Caddick MX et *al.*, (1998) and Salter MG et *al.*, (1998).

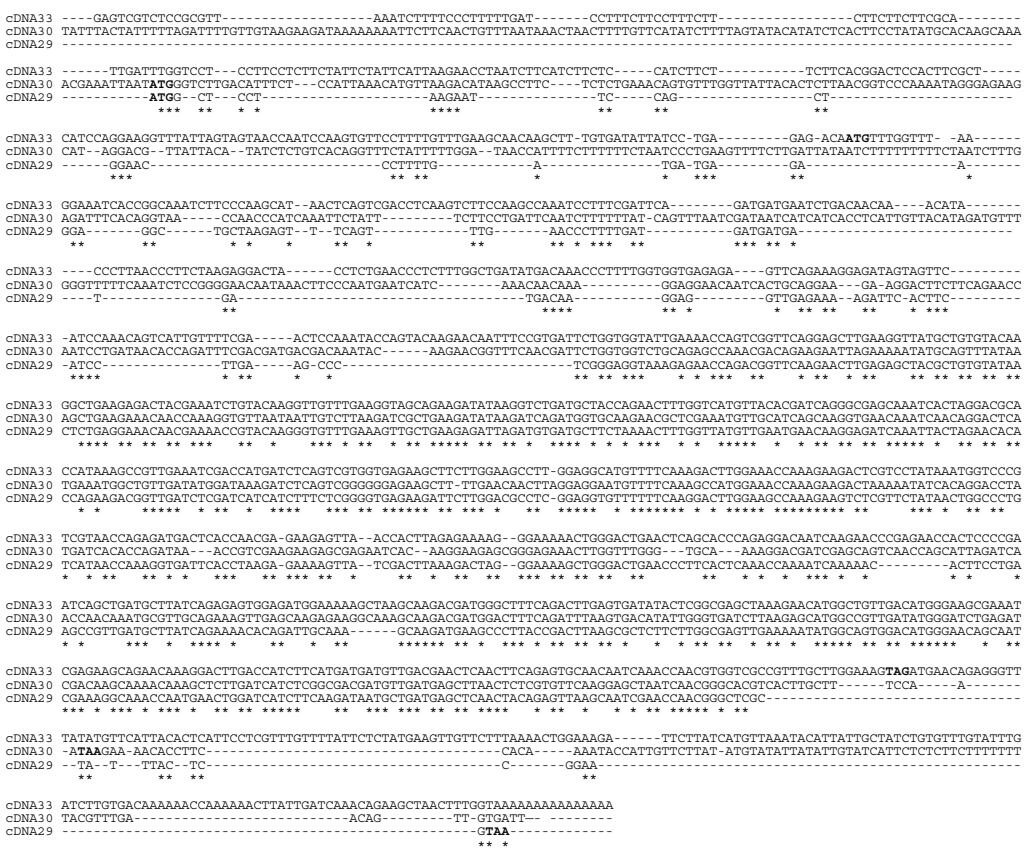
α-amylase activity test

Different dilutions of α-amylase solution (2 mg / ml) from Bacillus ap. (Sigma A 6380: 1'500 - 3000 units / mg) were put on a Petri plate with 1/2 MS containing 2% starch (Merck), incubated for 6h and colored with Lugol (0.02% I_2 and 0.06% sodium iodide). For the measurement of α-amylase activity, *A. thaliana* tissues were harvested and ground at 4°C with a mortar and a pestle in 2 ml per g fresh weight of 10mM acetate buffer pH 4.8 containing 20 mM $CaCl_2$. An adequate volume of the homogenate was added to 1 ml of 0.1% starch (Merck) and after different incubation time the reaction was stopped with Lugol. The degradation of starch was measured at 620nm with a spectrophotometer as described by Jones et *al.*, (1967).

Figure 1 **Sequence alignment of AtSNAP, AtSNAP30 and AtSNAP29**

A) The nucleotide sequence alignment of the AtSNAP33, AtSNAP30 and AtSNAP29 cDNAs done by the clustal X software. The underlined part of the cDNAs was amplified by PCR and used as a probe for northern blot analysis. The asterisks represent identical nucleotides. The translation start and stop codon are marked in bold.
B) The amino sequence alignment of AtSNAP33, AtSNAP30 and AtSNAP29 done by the clustal X software. Sequence identity is indicated by asterisks and homology by dots.

A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cDNA30 TATTTACTATTTTTAGATTTTGTTGTAAGAAGATAAAAAAAATTCTTCAACTGTTTAATAAACTAACTTTTGTTCATATCTTTTAGTATACATATCTCACTTCCTATATGCACAAGCAAA
cDNA29 ----------
cDNA33 ------TTGATTTGGTCCT---CCTTCCTCTTCTATTCTATTCATTAAGAACCTAATCTTCATCTTCTC-----CATCTTCT----------TCTTCACGGACTCCACTTCGCT-----
cDNA30 ACGAAATTAATATGGGTCTTGACATTTCT---CCATTAAACATGTTAAGACATAAGCCTTC----TCTCTGAAACAGTGTTTGGTTATTACACTCTTAACGGTCCCAAAATAGGGAGAAG
cDNA29 -----------ATGG--CT---CCT---------------------AAGAAT---------------TC-----CAG----------------CT----------
cDNA33 CATCCAGGAAGGTTTATTAGTAGTAACCAATCCAAGTGTTCCTTTTGTTTGAAGCAACAAGCTT-TGTGATATTATCC-TGA---------GAG-ACAATGTTTGGTTT- -AA------
cDNA30 CAT--AGGACG--TTATTACA--TATCTCTGTCACAGGTTTCTATTTTTGGA--TAACCATTTTCTTTTTTCTAATCCCTGAAGTTTTCTTGATTATAATCTTTTTTTTTCTAATCTTTG
cDNA29 ------GGAAC-----------------------------CCTTTTG-----------A---------------TGA-TGA---------GA-------------------A-------
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cDNA30 AGATTTCACAGGTAA-----CCAACCCATCAAATTCTATT---------TCTTCCTGATTCAATCTTTTTTAT-CAGTTTAATCGATAATCATCATCACCTCATTGTTACATAGATGTTT
cDNA29 GGA-------GGC------TGCTAAGAGT--T--TCAGT-----------TTG-------AACCCTTTTGAT----------GATGATGA-------------------------
cDNA33 ----CCCTTAACCCTTCTAAGAGGACTA------CCTCTGAACCCTCTTTGGCTGATATGACAAACCCTTTTGGTGGTGAGAGA----GTTCAGAAAGGAGATAGTAGTTC---------
cDNA30 GGGTTTTTCAAATCTCCGGGGAACAATAAACTTCCCAATGAATCATC--------AAACAACAAA----------GGAGGAACAATCACTGCAGGAA---GA-AGGACTTCTTCAGAACC
cDNA29 ----T---------------GA---TGACAA----------GGAG---------GTTGAGAAA--AGATTC-ACTTC---------
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cDNA29 AGCCGTTGATGCTTATCAGAAAACACAGATTGCAAA------GCAAGATGAAGCCCTTACCGACTTAAGCGCTCTTCTTGGCGAGTTGAAAAATATGGCAGTGGACATGGGAACAGCAAT
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cDNA29 CGAAAGGCAAACCAATGAACTGGATCATCTTCAAGATAATGCTGATGAGCTCAACTACAGAGTTAAGCAATCGAACCAACGGGCTCGC----------
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cDNA30 -ATAAGAA-AACACCTTC-----------------------------------CACA-----AAATACCATTGTTCTTAT-ATGTATATTATATTGTATCATTCTCTCTTCTTTTTTT
cDNA29 --TA--T---TTAC--TC-----------------------------------C-------GGAA----------
cDNA33 ATCTTGTGACAAAAAACCAAAAAACTTATTGATCAAACAGAAGCTAACTTTGGTAAAAAAAAAAAAAA
cDNA30 TACGTTTGA-------------------------ACAG---------TT-GTGATT-- --------
cDNA29 ----------GTAA------------

B


AtSNAP33 MFGLRKSPANLPKHNSVDLKSSKPNPFDSDDESDNK-HTLNPSKRTTSEPSLADMTNPFG
AtSNAP30 MFGFFKSPGN----N----K--LPN--ES---SNNKGGTITAGRRTSSEPIL--ITPDFD
AtSNAP29 ----------MAPK--------NSSWNPFDDEKEAAKSFSLN----PFD
AtSNAP33 GERVQKGDSSSSKQSLFSNSKYQYKNNFRDSGGIENQSVQELEGYAVYKAEETTKSVQGC
AtSNAP30 DD-----D----K----------YKNGFNDSGGLQSQTTEELEKYAVYKAEETTKGVNNC
AtSNAP29 DD---DDDKEVEKR---------FTSSLKPSGGKENQTVQELESYAVYNSEETTKTVQGC
AtSNAP33 LKVAEDIRSDATRTLVMLHDQGEQITRTHHKAVEIDHDLSRGEKLLGSLGGMFSKTWKPK
AtSNAP30 LKIAEDIRSDGARTLEMLHQQGEQINRTHEMAVDMDKDLSRGEKLLNNLGGMFSKPWKPK
AtSNAP29 LKVAEEIRCDASKTLVMLNEQGDQITRTHQKTVDLDHHLSRGEKILGRLGGVFSRTWKPK
AtSNAP33 KTRPINGPVVTRDDSPTRR-VNHLEKREKLGLNSAPRGQSRTREPLPESADAYQRVEMEK
AtSNAP30 KTKNITGPMIT-PDKPSKKSENHKEEREKLGLG--AKGRSSSQPALDQPTNALQKVEQEK
AtSNAP29 KSRSITGPVITKGDSPKRK-VIDLKTREKLGLNPSLKPKSKT---LPEAVDAYQKT--QI
AtSNAP33 AKQDDGLSDLSDILGELKNMAVDMGSEIEKQNKGLDHLHDDVDELNFRVQQSNQRGRRLL
AtSNAP30 AKQDDGLSDLSDILGDLKSMAVDMGSEIDKQNKALDHLGDDVDELNSRVQGANQRARHLL
AtSNAP29 AKQDEALTDLSALLGELKNMAVDMGTAIERQTNELDHLQDNADELNYRVKQSNQRARYLL
AtSNAP33 GK
AtSNAP30 SK
AtSNAP29 RK

RESULTS

Spontaneous formation of lesions and defence responses are triggered in the mutant

The *atsnap33* mutants grown on ½ MS medium showed no differences compared to the wild type WS plantlets until 7 days after germination. Then the formation of spontaneous necrosis on several parts on the cotyledons was observed. The necrosis spread on the whole cotyledons during the next 2 to 3 days (Figure 2A). The developing first true leaves were not yet affected by this necrosis formation. Once the cotyledons were completely necrotic and brown the necrosis also formed on the first true leaves. In contrast to the cotyledons where site of necrosis formation seemed random, the necrotic area in first true leaves began always at the tip and spread to the rest of the leaf. Eleven days after germination necrosis were also observed on the stem. From this time point on the formation of the necrosis was faster than the formation of new plant tissue, so that the *atsnap33* were dwarfs compared to the wild type WS plants at the same age. Three to four week after germination the mutants were dead.

Necrosis formation on the cotyledons 7 days after germination was visualized by trypan blue staining. Trypan blue stains dead plant cells dark blue. In living cells trypan blue does not form a complex with the cytoplasme and the cells are transparent, after destaining with chloral hydrate. After microscopical analysis, several parts of the cotyledons showed large blue areas corresponding to the brown areas observed under the binocular in unstained cotyledons (Figure 2B).

Reactive oxygen intermediates (ROIs) are typical markers for programmed cell death produced by the oxidative burst. The formation of ROI is one of the first responses to PCD, before lesion get visible. The *in situ* production of H_2O_2 in 7 day-old plantlets was detected by infiltration of diaminobenzidine (DAB) which forms a brownish precipitate in the presence of H_2O_2 and endogenous peroxidases. The cotyledons of 7 day old *atsnap33* plantlets with visible lesions were nearly completely brownish compared to the wild type indicating a high level of H_2O_2. The first true leaves without any visible necrosis at this time showed a brownish precipitate at the tip of the leaf. At this site the formation of a necrosis was observed 4 days later (Figure 2C).

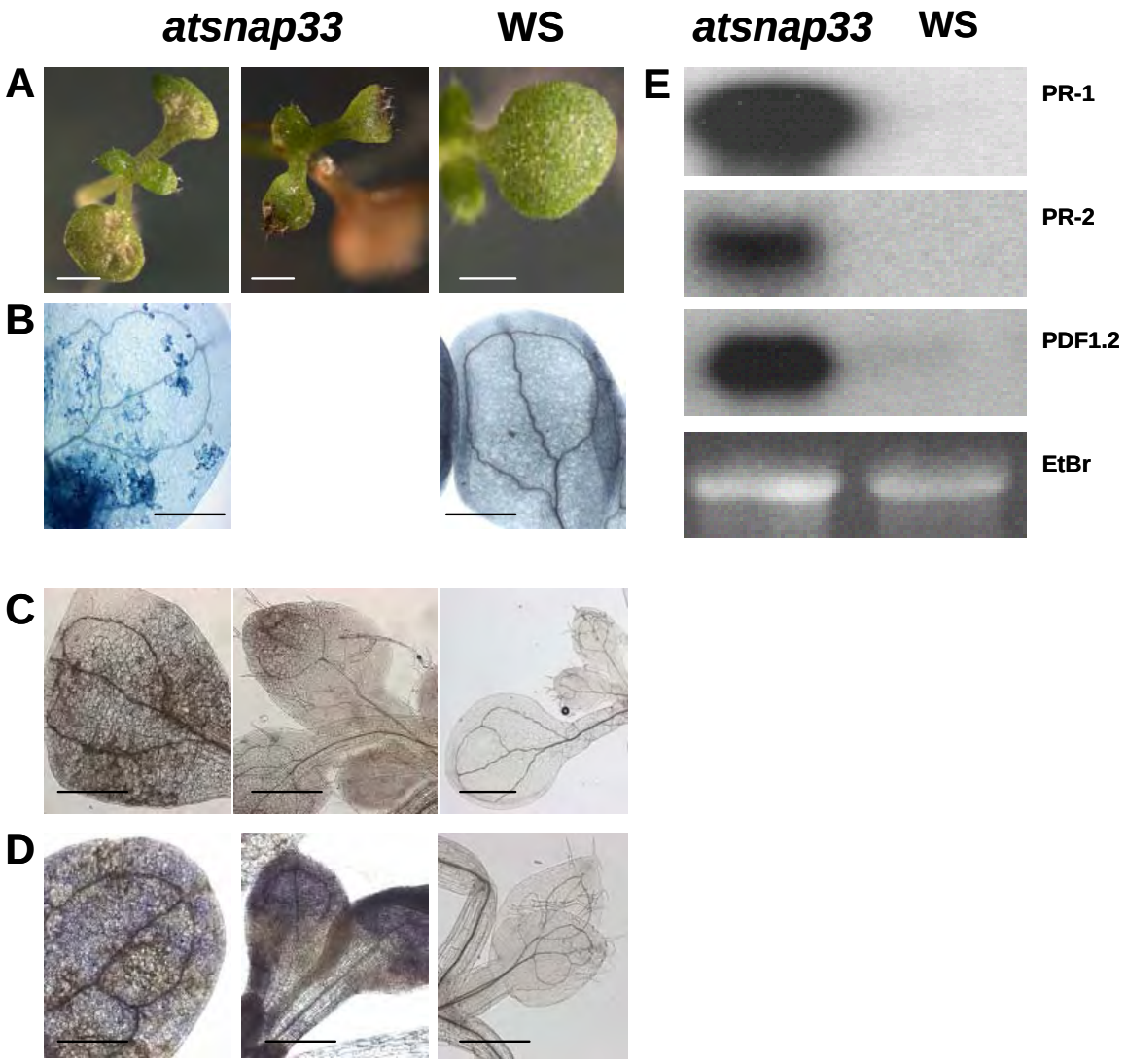

Figure 2 **The formation of lesions and ROIs in *atsnap33* mutant and the upregulation of defence genes**
A Images of the *atsnap33* mutant 7 days (left) and 11 days (middle) after germination showing the formation of lesions on the cotyledons and the first leaves compared to the wild type WS plant showing no lesions (right). Bar corresponds to 1mm
B Trypan blue coloration of 7 day old *atsnap33* mutant plantlet (left) show several initiations of lesion formation on the cotyledons. A wild type plantlet is shown on the right
C Seven 7 days old cotyledons of the *atsnap33* mutant (left) show a strong brownish coloration after infiltration of DAB which visualize the presence of H_2O_2, compared to the wild type (right). 11 days after germination the first leaves of the *atsnap33* mutant (middle) have a strong production of H_2O_2 in the tip of the leaf.
D The production of O_2^- was detected by NBT infiltration which forms a dark blue precipitate in the presence of O_2^-. Cotyledons of *atsnap33* 7 day and the first leaves 11 day after germination were coloured blue compared to the wild type of the same age.
A - D Bar correspond to 1mm
E The expression of the defence genes PR-1, PR-2 and defensin PDF1.2 are induced in the 7 day old *atsnap33* mutant and not abundant in the wild type of the same age shown by Northern blot analysis.

The detection of O_2^- another reactive oxygen intermediate was visualized by infiltration of nitroblue tetrazolium (NBT). In 7 day-old *atsnap33* mutant plantlets a blue precipitate was observed in the cotyledons indicating the presence of O_2^-. The first leaf contained detectible amounts of O_2^- compared to the wild type WS plantlets after 11 days (Figure 2D). NBT turns from a pale yellow solution to an insoluble blue / purple formazan product in the presence of O_2^- (Doke and Ohashi, 1988).

Some lesion mimic mutants show induced defence responses in the absence of a pathogen. In the *atsnap33* mutant the defence responses were up regulated. The pathogenesis-related proteins PR-1 and PR-2 as well as the defensin PDF1.2 were expressed in 7 days old *atsnap33* plantlets as shown by Northern blot analysis (Figure 2E).

The formation of lesion in the *atsnap33* mutants could not be inhibited by antioxidant substances such as diphenyleneiodonium (DPI), an inhibitor of the plasma membrane located NADPH oxidase (Hancock and Jones, 1987), nor ascorbatic acid, KI, butylated hydroxy toluene (BHT), nor the reductant dithiothreitol (DTT) (Bethke and Jones, 2001) (data not shown).

The homologues AtSNAP29 and AtSNAP30 were not expressed in the *atsnap33* mutant.

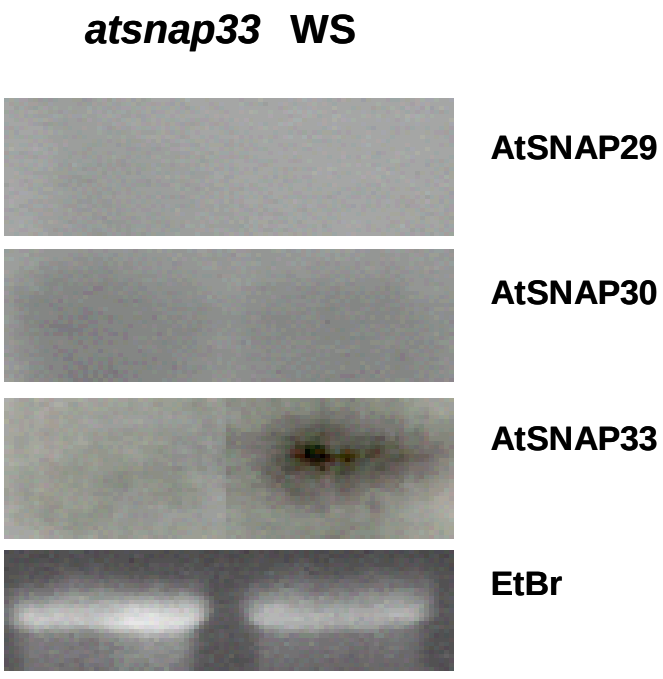

Figure 3 **Expression of AtSNAP33, AtSNAP29 and AtSNAP30 in the *atsnap33* mutant**
Total RNA from the *atsnap33* mutant and wild type (WS) was analysed by RNA blot hybridisation using probes for *AtSNAP33, AtSNAP30* and *AtSNAP29* as indicated in Figure 2A. Equal loading is shown by the agarose gel stained with ethidium bromide.

AtSNAP33 belongs to a small gene family of three members together with AtSNAP29 and AtSNAP30. Based on sequence analysis at the amino acid level, the identity between the 3 homologues is 55% - 62%. The C-terminal part of the homologues showed a higher degree of identity than the N-terminal region (Figure 1B).

The *atsnap33* plantlets showed no visible phenotype before 7 days. This could mean that the 2 homologues AtSNAP29 and AtSNAP30 could functionally compensate for the lack of AtSNAP33. As expected,

AtSNAP33 was not expressed in the *atsnap33* mutants compared to the wild type where a weak expression was detected by Northern blot analysis. However, the homologues AtSNAP29 and AtSNAP30 were not expressed in the atsnap33 or wild type seedlings at this developmental stage (Figure 3).

Root growth was decreased in the mutant but the roots show no necrosis

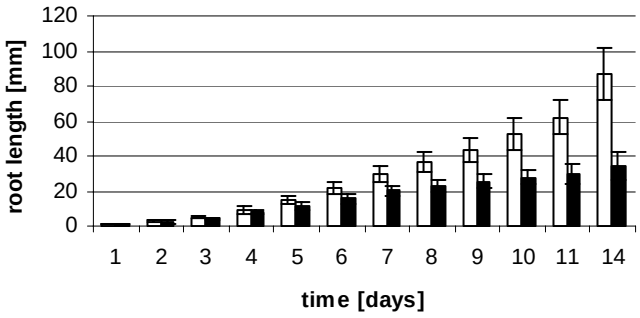

Figure 4 **Root growth of *atsnap33* mutants and wild type**
Roots from *atsnap33* mutant and wild type plantlets grown *in vitro* were measured daily. The mean value +/- standard deviation of the root length of 15 wild type (white bars) and *atsnap33* mutant (black bars) plants is shown.

The roots were not affected by the formation of necrosis during the whole life time, of the *atsnap33* mutant i.e. 3 – 4 weeks. The only measurable differences between the *atsnap33* and wild type roots was their growth rate. The roots of wild type and *atsnap33* plants placed on Petri plates growed with the same rate for the first 5 to 6 days. Six to seven days after germination, the growth rate of the roots of the atsnap33 mutants decreased compared to the wild type. Eleven days after germination, the wild type roots were 60mm long compared to the 30 mm long roots of the mutant (Figure 4). Fourteen days after germination the roots of the mutant and the wild type started to branch making the measurement of their length difficult.

A barley α-amylase expressed in *Arabidopsis* can be used as a marker for secretion

In barley, α-amylases are expressed in the aleurone layer of the grain and degrade the starch stored in the endosperm during germination of the grains. Starch is a

mixture of glucans composed of α-amylose, a linear polymer of glucose residues linked by α(1-4) bounds and amylopectin. The α-amylase randomly hydrolyses all the α(1-4) bounds except its outermost bonds and those next to branches (Voet and Voet, 1990). I_2IK stains the long branches of starch dark violet. Starch hydrolysed by α-amylase has no long chains anymore and is not stained anymore by I_2IK. A 1/10, 1/50, 1/100, 1/500, 1/1000 dilution serie from an α-amylase stock solution of 2 mg / ml was incubated for 6h on a 1/2 MS medium containing 2% starch. After staining with I_2IK the surface of the halo was measured and found to show a linear relationship with the amount of α-amylase in the medium (Figure 5B).

This assay allow to quantify the amount of secreted α-amylase as a marker for

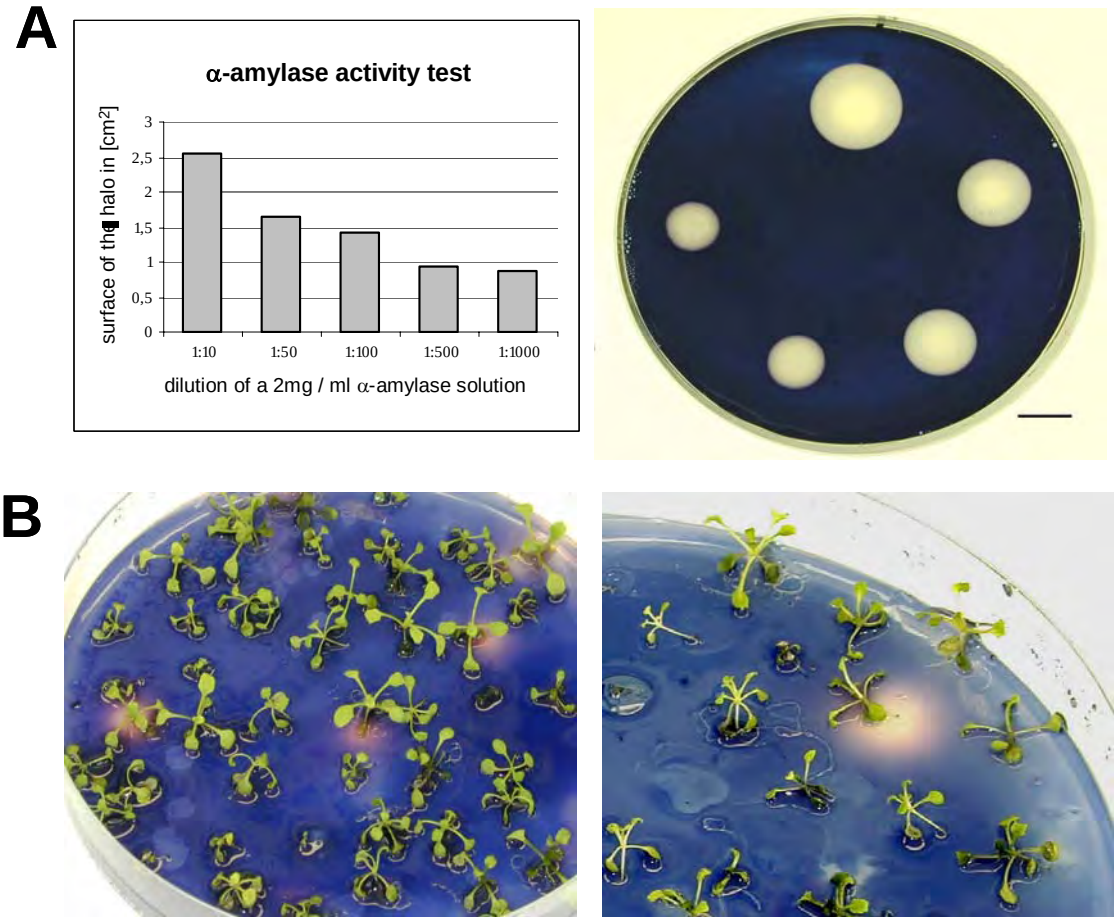

Figure 5 **Detection of barley α-amylase activity secreted by transgenic *Arabidopsis* plantlets on Petri plates**

A A 1/10, 1/50, 1/100, 1/500, 1/1000 dilution serie of an α-amylase solution (2 mg / ml) as placed on 1/2 MS medium containing 2% starch, incubated for 6h and stained with a lugol (I_2/IK) solution. The graph show the correlation of the surface of the white halo formed by the different α-amylase concentration. The photo on the right was taken after the petri dish was stained with lugol. Bar correspond to 1 cm.

B *Arabidopsis thaliana* ecotype Columbia was transformed with barley α-amylase Clone E under the control of the CaMV 35S promoter. The T1 generation was grown on 1/2 MS medium containing 2% starch and stained with lugol 2 weeks after germination. Plants with a white halo are successfully transformed and secrete α-amylase.

secretion in α-amylase transformed *Arabidopsis* plants. The seeds of the T1 generation of *Arabidopsis* plants transformed with the α-amylase cDNA clone E or pM/C were sterilized and grown for 14 days on 1/2 MS mediun containing 2% starch. The plates were stained with I_2IK. Successfully transformed plants were able to secrete the α-amylase in the medium and a white halo surrounded these plants on the Petri plate (Figure 5C). They were kept to generate homozygote lines.

The level of expression of AtSNAP33 is not increased in transgenic plants expressing high amounts of α-amylase

Arabidopsis thaliana accession Columbia plants were transformed with the barley α-amylases cDNA Clone E or pM/C under the control of the cauliflower mosaic virus 35 S promoter for constitutive expression in plants. The α-amylase expression and activity as well as the expression of AtSNAP33 were analysed in the different T1 lines (Figure 6).

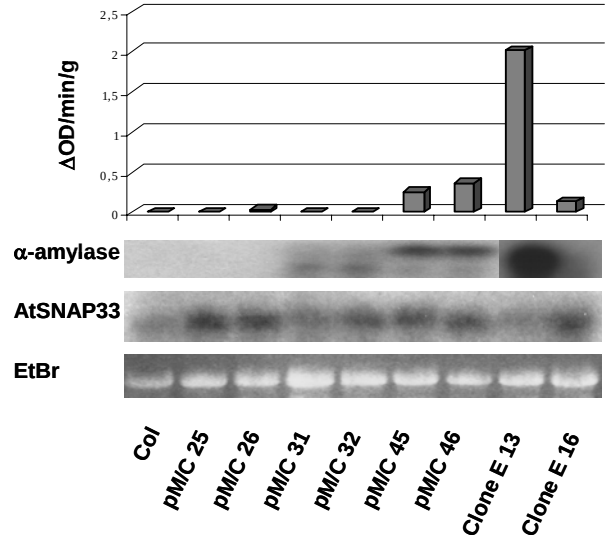

Figure 6 **Correlation between α-amylase activity and the expression of the AtSNAP33 gene in different lines of *Arabidopsis* plants transformed with barley α-amylase.**
The upper part of the figure represents the α-amylase activity of different lines of *Arabidopsis* plants transformed with barley α-amylase cDNA Clone E or pM/C under the control of the CaMV 35S promoter. Total RNA from the same lines was also analysed by RNA blot hybridisation using probes for α-amylase pM/C, Clone E and AtSNAP33. The α-amylase activity correlated with the expression pattern of the α-amylase gene. AtSNAP33 is expressed in all transgenic lines but not in control Col plants. Equal loading is shown by the agarose gel stained with ethidium bromide.

The α-amylase activity test was performed on a total protein extract from leaves of the different T1 lines. The highest activity was measured in the line E13. There was no activity in leaves of control, untransformed Columbia plants. The level of α-amylase transcripts related to the activity measurements of the different T1 lines. The level of α-amylase transcripts were five to ten times higher in the line Clone E13 compared to the other transformed lines. AtSNAP33 transcripts were expressed in a similar level in T1

lines expressing different levels of α-amylase (Figure 6).

The T1 line pM/C 31 and pM/C32 show a smaller transcripts of the pM/C cDNA then the pM/C 45 and pM/C line 46. This could be an incomplete insertion of the cDNA in the genome of Arabidopsis after the transformation by *Agrobacterium*. Since they showed also no α-amylase activity measured.

The *atsnap33* mutant secretes α-amylase until 7 days but then secretion is highly reduced

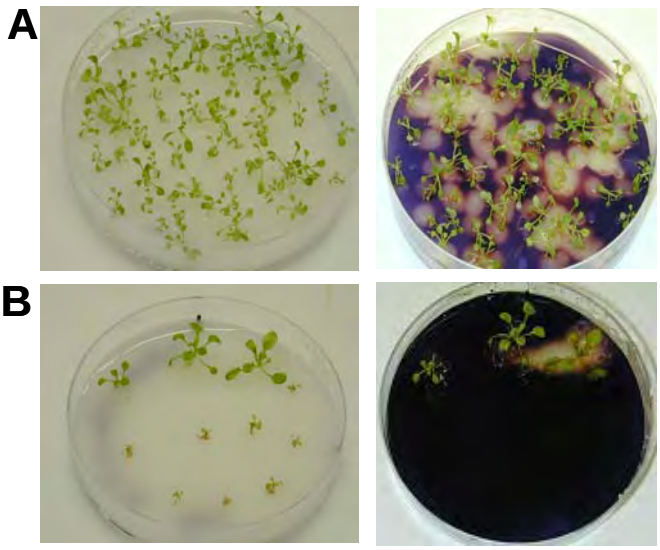

Figure 7 **The secretion of barley α-amylase in transgenic *Arabidopsis* is highly reduced in atsnap33 mutant 7 day after germination**

A *atsnap33* mutants expressing the barley α-amylase were grown in 1/2 MS medium during 7 days then they were transferred to new plates containing 1/2 MS with 2% starch.

B After another 7 days the plates were stained with lugol (photos on the right). The two well developed plants (left and middle) in the upper line of the Petri plate are untransformed wild type (WS) plants. The well developed plant on the right of the upper line is a wild type plant transformed with the barley α-amylase cDNA which expresses and secretes α-amylase as shown by the white halo. The six dwarf plants in the middle and lower line are *atsnap33* mutants transformed with the α-amylase gene but the α-amylase is not secreted anymore.

Arabidopsis plants transformed with barley α-amylase secrete α-amylase, which is easy to detect with the lugol staining and show no alteration of growth or development (see above). A plant population of heterozygote *atsnap33* mutant were transformed with the same barley α-amylase as used for the *A. thaliana* ecotype Columbia. The expression of the α-amylase in the T1 generation did not alter the phenotype of the atsnap33 mutant. Seeds of the T1 generation were sterilized and grown on 1/2 MS containing 2% starch. Seven day after germination when the mutant develop its specific phenotype, the medium was stained with lugol. Since the line was not yet homozygote for α-amylase only some of the wild type, heterozygote as well as *atnap33* homozygote mutants showed an α-amylase secretion activity (upper part of Figure 7). Two 7 day

old wild type plants and one 7 day old Columbia plant, expressing constitutively α-amylase were transferred to a new Petri dish containing 1/2 MS 2% starch medium as controls. Six *atsnap33* mutants recognized by their phenotype, and secreting during the first 7 days α-amylase in the medium were chosen and transferred to new Petri dishes. The plate was put back in the growth chamber for another 7 days. The plate with the 14 day old plants was stained with lugol. Only the Columbia plant expressing the α-amylase showed a white halo after staining. The six *atsnap33* mutant were not able to secrete the α-amylase anymore once they reached the age of 7 days (the lower part of Figure 7).

This preliminary experiment has to be repeated with the homogygote for α-amylase *atsnap33* mutant plants and additionally the genotype of each plant has to be verified by PCR. The data of these experiments indicate clearly the inhibition of secretion activity in atsnap33 mutant plants showed by the marker enzyme α-amylase.

Discussion

The „„knockout““ of the t-SNARE homologue AtSNAP33 leads to a dwarf phenotype and the plants die after 3 - 4 weeks. The development of the *atsnap33* mutant shows no visible differences compared to the wild type during the first 7 days after germination. However 7 days after germination necrotic spots were observed on the cotyledons which spread to the whole leaf and later to the whole plant except the roots. The necrotic spots were very similar to necroses formed by hypersensitive reaction (HR) but in the absence of a pathogen. There are other mutants with a lesion mimic phenotype in *Arabidopsis* (Dietrich *et al*., 1994). Some of these mutants produce an oxidative burst, the formation of reactive oxygen intermediates (ROIs) and trigger defence responses such as the expression of pathogenesis related proteins (PRs) similar to the response to a pathogen attack (for a review see Mittler and Rizhsky, 2000). In the *atsnap33* mutant we observed an increase of H_2O_2 and O_2^- (both ROIs) in the first true leaves before lesions were visible and the induction of defence mechanisms shown by the expression of PR-1, PR-2 and the plant defensin

PDF1.2. Interestingly, AtSNAP33 is induced in infected leaves (Peter Wick, Xavier Gansel and Liliane Sticher unpublished results).

Why the roots are not affected by lesion formation is not clear. This is surprising because we showed (Heese *et al*., 2001) that AtSNAP33 is highly expressed in roots especially in the root tips. Since the function and expression of the homologues of AtSNAP33, AtSNAP30 and AtSNAP29 are not known, we cannot exclude a redundancy of one of these homologues or another SNARE in the roots. The only effect which was measured in the roots of the *astnap33* mutant was a reduction of the growth rate compared to the wild type. This could be the cause of the reduced capability of vesicle fusion with the plasma membrane which is needed for the growth of the root cells and roots. Enquist and Niklas, (2002) developed a general allometric model showing a log-log linear correlation among 'below-ground' biomass (roots) and 'above-ground' biomass (leaves and stem). A reduction in above-ground biomass as observed in the *atsnap33* mutant would also explain the reduced growth rate of the roots compared to the wild type.

The strongest evidence that AtSNAP33 is involved in secretion come from the observations of the *atsnap33* mutant transformed with the α-amylase from barley. The inhibition of the secretion of α-amylase in the mutants correlates with the appearance of the mutant phenotype. During the first 7 days after germination when the mutant phenotype is not expressed, there was secretion of α-amylase. During the next 7 days the phenotype of *atsnap33* mutants appeared and the secretion of α-amylase in the MS medium is inhibited.
In transformed *A. thaliana* plants expressing and secreting constitutively α-amylase, there was surprisingly no correlation between the level of expression of AtSNAP33 and of α-amylase transcripts. It is not known if an increased expression of a secreted protein such as α-amylase provokes an increase of the machinery of secretion including possibly AtSNAP33 or if the machinery can accommodate higher levels of secretion without novel expression of its components. We isolated lines expressing 10 times more α-amylase than the lines presented in this work and it will be interesting to see if the expression of AtSNAP33 is increased in these lines.

We hypothesice based on the results presented that the secretion is functional only in the presence of AtSNAP33. In *atsnap33* mutant plants secretion is disturbed, essential components within the plant cell are not anymore transported. This lead to the dramatic disturbance which interferes with the PCD and lead to the lesion mimic phenotype of the mutants. The lack of expression of the phenotype during the first 7 days could be explained by the fact that the two homologues of AtSNAP33, AtSNAP30 and AtSNAP29 function to complement AtSNAP33.

Levine *et al*., (2001) described that the v-SNARE AtVAMP could prevent apoptosis in yeast and *Arabidopsis* cell. The expression of the proapoptotic mammalian protein Bax in yeast or in *Arabidopsis* induced PCD. The Bax-induced PCD was suppressed by transformation with AtVAMP, an *Arabidopsis* SNARE. AtVAMP also prevented H_2O_2 induced apoptosis in yeast as well as in *Arabidopsis* cells. These results are similar to the results presented with the *atsnap33* mutants, i.e. perturbation in trafficking can regulate execution of PCD.

Acknowledgement

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Characterization of the potential AtSNAP33-interacting syntaxins AtSyp122 and AtSyp43 in *Arabidopsis thaliana*

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Key-words: *Arabidopsis*, secretion, SNARE complex, syntaxin

ABSTRACT

Intracellular membrane traffic involves the binding of t-SNAREs on the target membrane and v-SNAREs on the vesicle membrane to form the SNARE core complex necessary for vesicle fusion. SNAREs are soluble *N*-ethylmaleimide-sensitive factor adaptor proteins receptors. A yeast two hybrid screen with AtSNAP33, a homologue of the neuronal SNARE SNAP-25 as a bait, led to the isolation of two not yet described syntaxins (t-SNARE) AtSYP122 and AtSYP43. Syntaxins are membrane proteins with a transmembrane domain at the C-terminus and a 60 amino acid long stretch the SNARE-motif in the cytosolic part. AtSYP122 belongs to the subclass SYP1 and is highly homologous to two other members of this subclass; AtSYR1 (AtSYP121) an *Arabidopsis* homologue of tobacco NtSYR1 which is involved in the ABA signalling pathway and KNOLLE (AtSYP111) necessary for proper phragmoplast formation during cytokinesis. AtSYP43 is in the subclass SYP4 together with the already described AtSYP41 and AtSYP42 better known as AtTLG2a and b which are localised in different parts of the TGN in plant cells. The transcripts of *AtSyp122* were expressed constitutively in roots but not in leaves. However *AtSyp122* transcripts were expressed in leaves after inoculation with the pathogens *Peronospora parasitica* and *Plectosporium tabacinum*. No transcripts of *AtSyp43* were found in these conditions. Interestingly, transcripts of *AtSyp43* were only found in the knock-out mutant *atsnap33*. To learn more about the function of these two syntaxins sense and antisense constructs of both *AtSyp122* and *AtSyp43* were made and used to transform *Arabidopsis thaliana* plants.

INTRODUCTION

The secretory pathway is a complex endomembrane system essential for all eukaryotic cells. It transports proteins to the extracellular space or to the vacuole. Proteins containing a signal peptide for entry in the endoplasmic reticulum (ER) are synthesized at ER and transported in the lumen of this organelle. They are then transported by vesicles to the Golgi apparatus. After moving through the stacks of the Golgi apparatus these proteins arrive in the *trans*-Golgi network (TGN). At the TGN, depending on the presence of an additional targeting signal they are sorted into vesicles destined for several organelles including the prevacuolar compartment (PVC), lytic vacuole, protein storage vacuole, (PSV) or for secretion to the plasma membrane (PM). In contrast to vacuolar proteins, secreted proteins do not have an additional targeting signal. Thus, secretion to the extracellular space is the default pathway. Transport between the organelles of the secretory pathway occurs by budding of vesicles from a donor membrane and fusion with an acceptor membrane. The fusion of the appropriate vesicles with the correct target membrane is of outmost importance, otherwise the cell will lose its compartmentalisation and collapse. A large group of proteins called soluble *N*-ethylmaleimide-sensitive factor adaptor proteins receptors (SNAREs) and their associated factors were described to be responsible for these fusions steps. The general hypothesis postulates that a membrane associated protein on the vesicle, the v-SNARE, interacts with another membrane associated protein on the target membrane, the t-SNARE (Rothman *et al*, 1996). Syntaxins, a family of t-SNAREs are anchored to the membrane via their C-terminal transmembrane domain. The N-terminus of the protein contains one SNARE motif, a ~60 amino acid long domain which is functionally important because it mediates the association of the SNAREs into core complexes. All SNAREs share a homologous domain of the SNARE motif (Jahn, 1999). In neurons, SNAP-25 a plasma membrane localized t-SNARE protein with two SNARE motifs, together with the v-SNARE synaptobrevin one SNARE-motif and the t-SNARE syntaxin1 one SNARE-motif are assembled into a four-helix SNARE bundle (SNARE core complex) (Jahn, 1999; McNew *et al.*, 2000).

Each step of the secretory pathway needs his own set of SNAREs and SNARE interacting factors (Rothman *et al.*, 1996).

Sequencing of the genome of *Arabidopsis* uncovered 24 syntaxins found by homology and analysis of common structural features. Sanderfoot et *al.*, (2000) classified these syntaxins in eight classes, based on their sequences and intron – exon structure. Only a few of them are well characterized like KNOLLE (AtSYP111) (Lukowitz *et al.*, 1996) which belongs to the SYP1 group or AtTLG2a/b both members of the SYP4 group (Bassham *et al.*, 2000). *Knolle* mutants are seedling lethal. KNOLLE is expressed only during cell division and is found at the phragmoplast (Lauber *et al.*, 1997). AtTLG2a/b (AtSYP41 and AtSYP42) localized at the *trans*-Golgi network are able to form a complex together with a Sec1-like/Munc-18 protein AtVPS45 (Bassham *et al.*, 2000).
The members of the SYP2 and SYP4 group share each 60% - 80% protein sequence identity. The gene disruption of the individual *Arabidopsis* syntaxins from these groups is lethal in the male gametophyte. This is surprising because the disruption of the yeast orthologs indicate that these syntaxins are not essential for growth in yeast. These results indicate that at least two syntaxin in the SYP2 and two from the SYP4 group serves an essential nonredundant function (Sanderfoot *et al.*, 2001)

AtSNAP33 was the first protein homologous to SNAP-25 described in *Arabidopsis thaliana*. AtSNAP33 belongs to a small gene family of three members, is ubiquitously expressed and is localized at the plasma membrane and to the forming cell plate (Heese *et al.*, 2001). *In vitro* binding assays were able to show that AtSNAP33 binds to the *Arabidopsis* syntaxin KNOLLE (Heese *et al.*, 2001) and to the tobacco syntaxin NtSYR1 (Kargul *et al.*, 2001) which was shown to be involved in the signalling pathway of ABA (Leymann *et al.*, 1999).
Plants respond to bacterial, fungal and viral pathogen attack by the synthesis of several pathogenesis-related (PR) proteins including PR-1 (van Loon et *al*, 1999). Many of them are synthesized at the ER and transported to the extracellular space by the secretory pathway. *Arabidopsis thaliana* accession Columbia infected with *Peronospora parasitica* isolates Noco and Emwa, *Plectosporium tabacium* or *Botrytis cinerea* show expression of PR1 (Thomma *et al.*, 2001).
A yeast two hybrid screen with AtSNAP33 as a bait resulted in the isolation of two not yet described syntaxins in *Arabidopsis thaliana* (Gansel and Sticher, personal communication). Based on their sequence one syntaxin belongs to the SYP1 group and is named AtSYP122 the other belongs to the SYP4 group and is named

AtSYP43 (Sanderfoot *et al.*, 2000). In this report we analysed the expression of *AtSyp122* and *AtSyp43* and made transgenic plants to try to find the biological function of these syntaxins.

MATERIALS AND METHODS

Plant material and bacterial strains

A. thaliana accession *Columbia*

A. thaliana ecotype Wassilewskija (WS)

ΔAtSNAP33 EFS396 (T-DNA insertion line obtained from the Versailles T-DNA collection (ecotype WS)

E.coli XL1-blue (resistant to 10 μg/ml tetracycline) and DH5α

Agrobacterium tumefaciens strain GV3101 (pMP90 helper plasmid, resistant to 25 μg/ml gentamycin, resistant to 100 μg/ml rifampicin)

Kits and Enzymes

For subcloning of PCR fragments the pGEM®-T easy vector system (Promega) was used. High quality plasmid DNA preparation were done using the following kits Machery & Nagel Midi Kit; Qiagen Mini Prep; Promega Midi Prep Wizard Plus kit. For the purification of DNA fragments from agarose gels the Qiagen gel extraction kit and Machery & Nagel Nukleobond kit were used.

All restriction endonucleases were provided by Gibco BRL and New England Biolabs and used as described in manufacturers instructions.

General molecular biology techniques were performed according to standard protocols described in Sambrook *et al.*, (1989).

Plant growth conditions and seed sterilisation

Arabidopsis plants sowed on soil and vernalized in the dark at 4°C for 4 - 5 days were grown in growth chambers under short day conditions (12 h light / 12 h dark) at 20°C. For in vitro culture the seeds were washed briefly with 70% ethanol and sterilised for 20 min with 2.5 % bleach containing 0.05 % Triton X-100. The bleach was removed with 5 washes with sterile water then the seeds were placed on ½ MS (Murashige and Skoog, 1962) with 1% sucrose and 0.7% agar. After vernalisation the plants were grown in the same conditions as in soil unless otherwise stated.

The knock-out mutant *atsnap33* resulted in a lethal dwarf phenotype. Seven days after germination the mutants show the formation of spontaneous and spreading lesions. At the age of 3 to 4 weeks the plantlets die. *Atsnap33* plants are not fertile and have to be maintained in the heterozygote stage. From heterozygote plants which were first tested by PCR according to the protocol of Heese *et al.*, (2001), seeds were harvested and grown on a selective ½ MS medium with 50 μg / ml kanamycin. Fifty % of the population of the seedlings are heterozygotes. They are resistant to kanamycin and survive. Twenty-five % of the population are wild type. They are kanamycin sensitive and will die and 25% correspond to the homozygote mutants. They are resistant to kanamycin but will die after 3 – 4 weeks. The mutant atsnap33 were selected 7 days after germination when the formation of the lesions were observed. They were analysed and compared to the wild type WS plants.

For *in vitro* root culture sterile 10-day-old plantlets grown on 0.38% B5 Gamborg salts (Flow Laboratories) containing 1% sucrose and 1% agar and adjusted to pH 5.5 were transferred to liquid medium (0.46% MS salts, 0.018% KH_2PO_4, 0.02% myo-inositol, 0.001% thiamin, 0.0001% pyridoxin, 0.0001% biotin, 0.0002% glycin, 0.0001% nicotinic acid, 0.00005% folic acid, 3% sucrose, adjusted to pH 5.8) where they were grown for 3 weeks in the dark. Then the roots were cut and transferred to the same liquid medium and grown in the dark. Every 4 weeks, the roots were divided in 4 parts and each part was transferred to a fresh liquid medium.

Bacterial growth conditions

The different strains of *E. coli* were grown in liquid medium (Miller's Luria broth base, Sigma) under continuous shaking or in Petri plates on Luria Broth with 1% agar at 37°C. For the selection of the transformed bacteria the adequate antibiotics were used.

Probes for Northern blot analysis of *AtSyp122* and *AtSyp43*

The set of primer 5'CCA TAT TTG ACT CGT CAA TAT AAA TAC3' and 5'TTG TTA CCA CCG TGG CAA CTT CC3' was used to amplify a specific probe from the cDNA *AtSyp122* by PCR. The 234bp PCR fragment corresponding to the 5' region of the cDNA, was cloned in the T-cloning sites of pGEM®-T easy vector (Promega). For a specific probe against *AtSyp43* the set of primer 5'AAG GAT CCC CAC AAG GAG ATC CCT C3' and 5'TTC CAT GGA CAG TTC TAT ATG ATC TCT TC3' was used to amplify a 335bp fragment corresponding to the 3' non-coding region from the *AtSyp43* cDNA by PCR which was cloned in the pGEM®-T easy vector. (Figure 1A and 1B)

Inoculation with *Peronospora parasitica* isolates Noco and Emwa and *Plectosporium tabacinum*

3 week old *A. thaliana* plants, grown in pots (20 - 30 plants per pot) were inoculated by spraying with a conidial suspension (10^5 – 10^6 conidia / ml sterile tap water) of *Peronospora parasitica* isolate Noco or Emwa prepared as described (Mauch-Mani and Slusarenko, 1994). After the treatment, the plants were kept in a growth chamber under short day conditions, high air humidity and 18°C. The control plants were treated only with water.

Plectosporium tabacinum was a kind gift of Dr. Brigitte Mauch-Mani (University of Neuchâtel, Switzerland) The fungus was kept on Petri plates on 3.9% potato-dextrose-agar medium (Difco laboratories). To obtain conidia, the surface was scraped with a steel eye and the conidia were suspended in water. Conidia were

counted under a microscope and a suspension of 2 x 10^6 conidia / ml was prepared in water and sprayed onto 3 week-old plantlets grown in pots containing 20-30 plantlets / pot.

RNA preparation, RNA blot analysis and probes

For RNA extraction, plant tissues were harvested, frozen and pulverized in liquid nitrogen. One volume of 2 M Tris pH 8 containing 0.5 M EDTA pH 8 and 20% SDS (v:v:v; 1:2:1) at 95°C was added, followed by 1 volume of saturated phenol:chloroform:isoamyl alcohol (v:v:v; 25:24:1) at 40°C. The two phases were separated by centrifugation, the aqueous phase containing the RNA was mixed with one volume of chloroform and separated by centrifugation. The RNA was precipitated with 1 volume of 6 M LiCl for at least 2 h or overnight at 4°C, washed with 70% EtOH and resuspended in water. For RNA blot analysis, 6μg of total RNA were applied on a 1% formaldehyde-agarose gel, separated and transferred to a nylon membrane (Hybond-N Amersham) as described by Sambrook et *al.*, (1989). The membranes were hybridised overnight at 65°C with a DNA probe made by random primed labelling in the presence of α-32P dCTP with the RadPrimed DNA Labeling System according to manufacturer's instructions (Life Technologies or Promega). The membranes hybridised with all probes described, were washed subsequently for 5 min with 2 x SSC containing 0.1% SDS and then 3 times for 20 min with 0.2 x SSC containing 0.1% SDS at 65°C before exposing to X-Omat film (Kodak) (Zimmerli *et al.*, 2000)

Construct encoding the fusion protein GST::AtSYP43p

The plasmid pGEX-Syp43 was constructed using the pGEX-5x-1 plasmid (Pharmacia Biotech). This plasmid encodes glutathione-S-transferase followed by a site for cleavage by the protease factor Xa and a multiple cloning site for in frame insertion of the gene of interest. The *AtSyp43* cDNA was amplified by PCR with the set of primers 5'AAT TCC CGG GTA TGG CGA CTA GG3' containing a *Sma*I site and 5'ATG CGG CCG CTC CAT CAC CAT ACC3' containing a *Not*I site. The *AtSyp43p*

cDNA (aa 1 – 307) lacking the transmembrane domain at the C-terminus was cloned in frame in the pGEX-5x-1 vector to get the fusion GST::AtSYP43, sequenced and verified.

Construct encoding the fusion protein GST::AtSYP122p

The set of primer 5'GTG GGA TCC CCA TGA ACG ATC TTC TC3' containing a *Bam*HI site and 5'ATG CGG CCG CTC TGC CTT AAC AAG CCG3' containing a *Not*I site was used to amplify the part of the *AtSyp122* cDNA encoding a cytosolic domain. Then the partial *AtSyp122* (aa 1 – 275) was cloned in frame in the pGEX-5x-1 vector (pGEX-Syp122), sequenced and verified.

Expression and purification of the fusion protein GST::AtSYP122p and GST::AtSYP43p

The *E.coli* strain DH5α was transformed with the plasmid containing the GST::*AtSyp122* construct. A bacterial culture was inoculated and grown until OD_{620nm} 0.6 was reached. The synthesis of the recombinant protein was induced with 1 mM IPTG (Isopropyl-1-β-D-thio-1 galactopyranosid, Eurobio). After 1 - 4 h, the bacterial cells were harvested by centrifugation. Three ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 1mM PMSF) and lysozyme (300 μg/ml final concentration) were added per 1 g of bacterial cells. After sonication of the cell suspension, it was centrifuged and the soluble as well as the insoluble fraction were analysed for the protein of interest by SDS-PAGE (Worrall, 1996).
The glutathione S-transferase::AtSYP122 fusion protein was purified with glutathione Sepharose 4B (Pharmacia Biotech) batch purification according to manufacturer's instructions.
To cleave the GST fusion protein the factor Xa was used. The GST::AtSYP122 was incubated with the factor Xa (Pharmacia) as indicated. Otherwise the manufacturer's instructions were followed.

Total protein extraction from *E. coli*, SDS-PAGE gel electrophoresis and Coomassie blue staining

One ml of *E.coli* suspension bearing the pGEX-Syp43, pGEX-Syp122 and pGEX-5x-1 grown in LB at 37°C with ampicillin (final concentration of 50 μg / ml) was centrifuged at 10'000g for 30 seconds. The supernatant was trashed and 30 μl of 3x SDS-PAGE buffer (Sambrook *et al.*, 1989) was added to the pellet. The solution was vortexed and heated at 95°C for 5 min. Fifteen μl of this total protein extract were loaded on a 13% SDS-PAGE gel after a 5 min centrifugation step at 10'000g. The proteins were separated by electrophoresis according to their sizes and stained by Coomassie blue as described (Sambrook *et al.*, 1989).

AlcA promoter::partialAtSyp122 constructs

The plasmid pACNSyp122p was constructed using the pACN plasmid (Caddick et *al.*, 1998), a kind gift from Dr. A. Greenland (Zeneca, Bracknell, Berkshire, UK) in which the chloramphenicol acetyltransferase (CAT) gene was replaced by a part of the *AtSyp122* cDNA as described below. The set of primer 5'AAA CTG CAG ATG AAC GAT CTT CTC TCC G3' and 5'TTT CTG CAG CTA GCA AGT CCA CTT CCG TG3' both containing a *Pst*I site was used to amplify *AtSyp122* without the transmembrane domain at the C-terminal (*AtSyp122p*) from the *AtSyp122* cDNA. *AtSyp122p* (aa 1 – 287) was sequenced and cloned in the *Pst*I site of pACN replacing the CAT gene. The reporter cassette AlcA::*AtSyp122p* was transferred in the *Hin*dIII site of the binSRNACatN plasmid (without AlcA::CAT) as is recommended by Caddick *et al.*, (1998) and Salter *et al*., (1998) (see Figure 7).

Sense and antisense constructs of *AtSyp122* and *AtSyp43* containing the bialophos resistance (BAR) gene conferring resistance to the herbicide BASTA (glufosinate ammonium)

The vector family pAOV2, pSOV2, pKMB and pSMB were constructed for quick production of transgenic *Arabidopsis* plants containing either sense or antisense

copies of *EST* clones obtained from the PRL2 library (Mylne *et al.*, 1998). These vectors were a kind gift from Dr. J. Botella (University of Queensland, Departement of Botany, Brisbare, Australia). The full length cDNA of *AtSyp122* cloned in the pSport plasmid (Life Technologies) was cloned in the *Eco*RI and *Bam*HI sites of pSOV2 for sense expression and pAOV2 for antisense expression.
The full length cDNA of *AtSyp43* cloned in the pSport plasmid (Life Technologies) was transferred first in the *Hin*dIII and *Sma*I sites of the pBluescript plasmid (Stratagene). Then, the cDNA was transferred in the *Hin*dIII and *Sac*I site of pSMB for sense expression and pKMB for antisense expression.

Sense and antisense constructs of *AtSyp122* and *AtSyp43* containing the nptII gene conferring resistance to kanamycin

For the sense expression of *AtSyp122*, the *AtSyp122* cDNA cloned in the pSport plasmid (Life Technologies) was transferred in the p2RT vector containing a double 35S promoter using the *Bam*HI and *Kpn*I sites. The 2x35S::*AtSyp122* fragment was transferred in the binary vector Bin 19 using the *Hin*d III site (Frisch *et al.*, 1995).
For the antisense expression of the *AtSyp122* the *AtSyp122* cDNA cloned in the pSport plasmid (Life Technologies) was transferred in antisense orientation in the p2RT vector using the *Sma*I site. The 2x35S::*AtSyp12*2 fragment was transferred in the binary vector Bin 19 using the *Hin*dIII site (Frisch *et al.*, 1995).
To overexpress the *AtSyp43* gene in *Arabidopsis* the *AtSyp43* cDNA cloned in the pSport plasmid (Life Technologies) was transferred in the p2RT vector using the *Sma*I site. The 2x35S::*AtSyp43* fragment was transferred in the binary vector Bin 19 using the *Hin*dIII site (Frisch *et al.*, 1995).
For the antisense transformation with *AtSyp43* the set of primer 5'AAG GAT CCT CGG ATA CTA TAA TCC CTG G3' containing a *Bam*HI site and 5'TTC CAT GGA CAG TTC TAT ATG ATC TCT TC3' containing a *Nco*I site was used to amplify the 3' non-coding region from nucleotide 1231 to nucleotide 1487 of the *AtSyp43* cDNA. This fragment (*AtSyp43p*) was cloned in antisense orientation in the *Bam*HI and *Nco*I sites of the p2RT vector. The 2x35S::*AtSyp43p* fragment was transferred in the binary vector Bin 19 using the *Hin*dIII site (Frisch *et al.*, 1995).

Floral dip transformation of *Arabidopsis* plants with *Agrobacterium tumefaciens*

For *Arabidopsis* transformation, the floral dip method (Clough and Bent, 1998) was used with slight modifications. *Agrobacterium tumefaciens* strain GV3101 was transformed by electroporation with the construct designed for plant transformation. Single colonies were used to inoculate a pre-culture in LB medium (containing 25 μg/ml gentamycin, 100 μg/ml rifampicin and the corresponding antibiotic used for construct selection) and grown overnight at 28°C on a shaker at 220 rpm. 5ml of the pre-culture were used to inoculate 500 ml LB medium (containing 25 μg/ml gentamycin, 100 μg/ml rifampicin and the corresponding antibiotic used for construct selection) and grown overnight at 28°C on a shaker at 220 rpm. The bacteria from a 500 ml culture were harvested by a 10 min centrifugation at 5000 g at RT and resuspended in the infiltration medium (5% sucrose, 0.05% Silwett L-77) until an OD_{600} of 0.6 was reached. Withered flowers and siliques were cut off from 4 to 6 week old plants so that only flower buds or lightly opened flowers were left over. The inflorescences of plants were dipped for about 30 seconds into the infiltration medium. After dipping the plants were laid horizontally into a tray and the tray was covered overnight with a plastic bag. When the plastic bag was removed, the plants were put upright and cultured as normal.

Selection of transgenic plants

All transgenic plants generated in the course of this work were co-transformed with a kanamycin, hygromycin B or bialophos selectable marker. For selection of transformants T1-seeds were sterilized and placed on selection plates (1/2 MS, 1% sucrose, pH 5.8, 50 μg/ml kanamycin, 35 μg/ml hygromycin B or 0.4% BASTA (glufosinate ammonium)). Resistant plants developped into green seedlings and were transferred to soil after the first true leaves appeared.

A

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AtSyr1      ----------CTACGTCAGGG---ATTCA---------------TTCCGATCTGAA-ATCTCTCT---CTAGATTTCTCT--ATTTT-----TCGAA----TTTTAA---ATGAACGATT
Syp122      CCATATTTGACT-CGTCAATATAAATACAATTGTAACAAACAGATTTTGATCAAAAGATTTTACTTTTCTCAATTCCTTTCAACTTTCAAAATCGAAGATCTCTTAAGCCATGAACGATC
                      ** *****     ** **              **  ****  ** ** *  **   **  *** ** *  * ***     *****    * ****   *********

AtSyr1      TGTTTTCCAGCTCATTCTCTCGCTTCCGCAGCGGA-GAACCATCCCCTCGCCGAGACGTTGCCGGCGGTGGCGACGGAGTTCAGATGGCGAA----TCCCGCGGGATCAACC--GGTGGT
Syp122      TTCTCTCCGGCTCGTT-TAAAACCTCCGTCGCGGACGGTTCATCGCC--GCCG---CACTCACA-CAACATCGA--GATGTCA-AAAGCGAAAGTCTCCGGCGGAAGTTGCCACGGTGGT
            *  * *** **** ** *    * ****  ***** *   **** **  ****   *  *  *  *       ***  **  *** *  *****     *** **** *    **  ******

AtSyr1      GTGAACCTCGACAAGTTCTTCGAAGATGTTGAATCTGTGAAAGAAGAGCTAAAGGAGCTAGATCGGCTCAACGAAACACTCTCTTCATGTCACGAGCAGAGCAAGACGCTTCACAATGCT
Syp122      AACAACCTAGACACGTTCTTCCTCGACGTGGAGGTTGTAAACGAAGACCTTAAAGAGCTAGATCGTCTCTGTCACAACCTCCGATCGAGCAACGAACAGAGCAAGACGCTTCATAACGCT
               ***** **** *******   ** ** **   *** ** ***** ** ** ********** ***    * *  ***   **  *  **** ***************** ** ***

AtSyr1      AAAGCCGTTAAAGATCTCCGGTCTAAAATGGACGGTGACGTTGGAGTCGCGTTGAAGAAGGCGAAGATGATTAAAGTTAAACTCGAGGCGCTAGATCGTGCCAATGCTGCTAATCGGAGT
Syp122      AACGCGGTGAAGGAGCTGAAGAAGAAGATGGACGCTGACGTCACTGCTGCGCTTAAGACGGCGAGGCGCTTGAAAGGAAACCTCGAGGCTTTAGATCGGGCTAATGAAGTCAACCGAAGT
            ** ** ** ** ** **   *   ** ******* ******    *  *** * **** ***** *    * ****  ** ********  ******* ** ****  *  ** ** ***

AtSyr1      CTCCCTGGCTGTGGACCTGGTTCTTCCTCCGATCGAACCAGGACCTCTGTCCTCAATGGTCTCAGGAAGAAATTGATGGACTCTATGGATAGTTTCAACCGATTGAGGGAGCTTATCTCG
Syp122      TTACCGGAAAGTGGACCGGGTTCGTCGTCGGACCGGCAAAGAACTTCGGTGGTTAACGGGTTGAGGAAGAAGCTGAAGGATGAGATGGAAAAATTTAGTCGAGTGAGAGAAACGATCACG
             * ** *   ******* ***** ** ** ** **    ** ** ** **  * ** **  * ********  *** ***    ***** *  ** *  *** **** **    *** **

AtSyr1      TCCGAGTATAGAGAAACTGTACAGAGGAGGTACTTCACCGTCACCGGCGAGAATCCGGATGAACGAACCCTAGATCGACTGATTTCCACTGGAGAGAGTGAGAGATTCTTGCAGAAAGCA
Syp122      AATGAGTACAAAGAGACGGTTGGGAGAATGTGCTTCACCGTCACCGGCGAATATCCCGACGAAGCTACCCTCGAGCGTCTCATCTCCACAGGTGAAAGTGAAACATTTTGCAGAAAGCG
               ***** * *** ** **   *** * ** ******************  **** ** ***   ***** ** ** ** ** ***** ** ** ***** * *** ***********

AtSyr1      ATACAAGAACAAGGAAGAGGAAGGGTGTTAGACACCATTAACGAGATTCAAGAAAGGCATGATGCGGTTAAAGACATTGAGAAGAATCTCAGGGAGCTTCACCAGGTGTTTCTAGACATG
Syp122      ATTCAAGAACAAGGTCGTGGTAGGATTTTGGACACTATTAGCGAGATACAGGAGAGGCACGACGCAGTGAAAGATATTGAGAAGAGTTTGAATGAGTTGCACCAAGTGTTTCTTGATATG
            ** ***********  * ** *** * ** ***** **** ****** ** ** ***** ** ** ** ***** ********** * * *  *** * ***** ******** ** ***

AtSyr1      GCCGTGCTGGTAGAGCACCAGGGAGCTCAGCTTGATGACATCGAGAGTCATGTGGGTCGAGCTAGCTCCTTTATCAGAGGCGGAACTGACCAGCTACAAACCGCTCGGGTTTACCAGAAG
Syp122      GCTGTGTTGGTTGAGCATCAGGGTGCACAGCTTGATGACATTGAAGGCAACGTCAAACGAGCTAACTCGCTTGTTCGGTCTGGTGCGGACCGGCTTGTTAAGGCACGGTTTTATCAGAAG
            ** *** **** ***** ***** ** *************** **  *  * **    ******** ***  ** * *    **  * **** ***    *  ** *** **** ******

AtSyr1      AACACGCGAAAATGGACATGTATTGCCATTAT--TATT-CTCATCATCATCATAACTGTTGTGGTTCTTGCTGTTTTAAAACCGTGGAACAACAGCAGTGGCGGCGGCGGCGGTGGTGGT
Syp122      AACACACGGAAGTGGACTTGCTTTGCCATTTTGCTATTGCTCATTATTGTGGTTTTAATTGTGGTGTTTACTGTT---AAGCCTTGGGAGAGTAATGGTGGGGGAGGTGGAGGTGCACCT
            ***** ** ** ***** **  ******** *  **** ***** **  *  *    ********  ** ******   ** ** *** * *  *   **** ** ** ** ****    *

AtSyr1      GGTGGGGGTACCACTGGAGGAAGTCAACCAAATTCAGGGACACCACCAAATCCTCCTCAGGCAAGGCGTCTATTGCGT TGAAGTTGAAGTTGAAGTTGAGTTTCGTTATTTGCATATATA
Syp122      CGTCAAGCTACTCCGGT------TCAAGC----TCAGC--CACCGCCACCTCCGGCTGTGAATCGGCGGCTACTACGC TAAA--TAATTTTTTTTTTGAGGTTCCCT------------
             **   * ***  * *       **** *    ****   **** ***  ***  **  *    **** *** * ** * **  * *  **    ***** ***  *

AtSyr1      TTCTTTCTTTGAAAAACCTTAT-TATCAAACCAGCTTTG-----TGTTACTACTTTCTACTGCTGGTTTGTTGTTAATCTCCCGTTTATTTGGTTTTTGTGAAAGAATTTAAAATGTGGG
Syp122      --CTGTCTATGCATGAGCATATCTAT-------GTTTTGAAGATTGTTTTTTATTT-TACAG-TATTTTGTCTGTAATGTC---TTTGTAATGTTTTGGT-----TGGTTAAACTCTGTG
              ** *** ** *  * * *** ***       * ****     ****  *  *** *** * *  *****   **** **   *** *   ***** **       ***** * ** *

AtSyr1      TTAGATGAGAAAATTAGTACAACATTCTCTTGTATCTATGTTTGCT------------
Syp122      T-ATAT-ATAATGCGATCACCACATTTCTTTGTTT-TATTATGGAAAAAAAAAAAAAA
            * * ** * **    *  ** *****   **** * ***  * *
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B

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AtSYP121        MNDLFSSSFSRFRSGEPSPRRDVAGGGDGVQMANPAGSTGGVNLDKFFEDVESVKEELKE
AtSYP122        MNDLLSGSFKTSVADGSSPPHSHNIEMSKAKVSGGS-CHGGNNLDTFFLDVEVVNEDLKE
                ****.*.**.   .. .** ..     . ..... . . ** ***.** *** *.*.***

AtSYP121        LDRLNETLSSCHEQSKTLHNAKAVKDLRSKMDGDVGVALKKAKMIKVKLEALDRANAANR
AtSYP122        LDRLCHNLRSSNEQSKTLHNANAVKELKKKMDADVTAALKTARRLKGNLEALDRANEVNR
                **** ..* *..*********.***.*..***.** .***.*. .* .******** .**

AtSYP121        SLPGCGPGSSSDRTRTSVLNGLRKKLMDSMDSFNRLRELISSEYRETVQRRYFTVTGENP
AtSYP122        SLPESGPGSSSDRQRTSVVNGLRKKLKDEMEKFSRVRETITNEYKETVGRMCFTVTGEYP
                *** .******** ****.******* *.*..*.*.** *..**.*** *  ****** *

AtSYP121        DERTLDRLISTGESERFLQKAIQEQGRGRVLDTINEIQERHDAVKDIEKNLRELHQVFLD
AtSYP122        DEATLERLISTGESETFLQKAIQEQGRGRILDTISEIQERHDAVKDIEKSLNELHQVFLD
                ** **.********* *************.****.***************.*.********

AtSYP121        MAVLVEHQGAQLDDIESHVGRASSFIRGGTDQLQTARVYQKNTRKWTCIAIIILIIIITV
AtSYP122        MAVLVEHQGAQLDDIEGNVKRANSLVRSGADRLVKARFYQKNTRKWTCFAILLLLIIVVL
                *****************..* **.*..*.*.*.*.* .**.**********.**..*.**...

AtSYP121        VVLAVLKPWNNSSGGGGGGGGGTTGGSQPNSGTPPNPPQARRLLR
AtSYP122        IVVFTVKPWESNGGGGGGAPRQATPVQAQP----PPPPAVNRRLLR
                .*. ..***....*****.   .*.  .**    ** *.  *****
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C

D

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AtSyp43       MATRNRTLLFRKYRNSLRSVRAPMGSSSSSTLTEHNSLTGAKSG-LGPVIEMASTSLLNP
AtSyp42       MATRNRTTVYRKHRDACKSARAPLSLSAS-----------DSFG--GPVIEMVSGSFSRS
AtSyp41       MATRNRTLLFRKYRNSLRSVRAPL-SSSS--------LTGTRSGGVGPVIEMASTSLLNP
              ******* ..**.*.. .*.***.  *.*             *  ******.* *. ..

AtSyp43       NRS-YAPVSTEDPGNS-RGTITVGLPPDWVDVSEEISVYIQRARTKMAELGKAHAKALMP
AtSyp42       NHSSYAPLNSYDPGPSSSDAFTIGMPPAWVDDSEEITFNIQKVRDKMNELAKAHSKALMP
AtSyp41       NRS-YAPISTEDPGTSSKGAITVGLPPAWVDVSEEISVNIQRARTKMAELGKAHAKALMP
              *.* ***... *** *  ...*.*.** *** ****.. **..* ** **.***.*****

AtSyp43       SFGDGKEDQHQIETLTQEVTFLLKKSEKQLQRLSAAGPSEDSNVRKNVQRSLATDLQNLS
AtSyp42       TFGDNKGIHREVEMLTHEITDLLRKSEKRLQMLSTRGPSEESNLRKNVQRSLATDLQNLS
AtSyp41       SFGDGKEDQHNIESLTQEITFLLKKSEKQLQRLSASGPSEDSNVRKNVQRSLATDLQLLS
              .***.*  ....* **.*.* **.****.** **. ****.**.************* **

AtSyp43       MELRKKQSTYLKRLRLQKE--DGADLEMNLNGSRYKA-EDDDFDDMVFSEHQMSKIKKSE
AtSyp42       MELRRKQSTYLKRLQQQKEGQDEVDLEFNVNGKMSRLDEEDELGGMGFDEHQTIKLKEGQ
AtSyp41       MELRKKQSTYLKRLRQQKE--DGMDLEMNLSRNRYRP-EEDDFGDMLN-EHQMSKIKKSE
              ****.*********. ***  *  ***.*.. .  .  *.*....*   ***  *.*...

AtSyp43       EISIEREKEIQQVVESVSELAQIMKDLSALVIDQGTIVDRIDYNIQNVASTVDDGLKQLQ
AtSyp42       HVSAERE REIQQVLGSVNDLAQIMKDLSALVIDQGTIVDRIDYNVQNVSTSVEEGYKQLQ
AtSyp41       EVSVEREKEIQQVVESVNDLAQIMKDLSALVIDQGTIVDRIDYNIENVATTVEDGLKQLQ
              ..* ***.*****. **..************************..**...*..* ****

AtSyp43       KAERTQRQGGMVMCASVLVILCFIMLVLLILKEILL
AtSyp42       KAERTQREGAMVKCATILLVLCLIMIVLLILKNILF
AtSyp41       KAERTQRHGGMVKCASVLVILCFIMLLLLILKEIFL
              *******.*.** **..*..**.**..*****.*..
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Figure 1 **Sequence alignments of *AtSyp122* and *AtSyp43* with other syntaxins**
A) cDNA alignment of *AtSyp122* with *AtSYR1*, the closest related syntaxin of the SYP1 subclass. The underlined part of the *AtSyp122* cDNA was amplified by PCR and used as a probe for northern blot analysis.
B) Protein alignment of AtSYP122 and AtSYR1. The asterisks indicate identity and double point and single point homology of the two sequence.
C) cDNA alignment of AtSyp43, AtSyp41 and AtSyp42 the other members of the SYP4 subclass. The underlined part of the AtSyp43 cDNA at the 3' end was amplified by PCR and used as a probe for northern blot analysis. The asterisks represent identical nucleotides. The translation start and stop codon are marked in bold.
D) Protein alignment of AtSYP41, AtSYP42 and AtSYP43. The asterisks indicate identity and double point and single point homology of the three sequences.

RESULTS

The two syntaxins AtSYP122 and AtSYP43 belong to the SYP1 and SYP4 subclasses

AtSYP122 and AtSYP43 isolated by a yeast two hybrid screen with AtSNAP33 as a bait were two novel not yet characterized syntaxins in *Arabidopsis thaliana*. Both syntaxins have the typical transmembrane domain at the C-terminal end found for AtSYP122 at aa 275 – 307 and for AtSYP43 at aa 307 – 330. Based on its sequence and according to the classification made by Sanderfoot *et al.*, (2000) AtSYP122 belongs to the first group of syntaxin SYP1. The subclass SYP1 has three subgroups SYP11, SYP12 and SYP13. AtSYP122 is in the subclass SYP12. The syntaxin closest to AtSYP122 is AtSYP121 better known as AtSYR1. They share 68% homology and 53% identity (see also the sequence alignment of AtSYP122 with AtSYR1 Figure 1A). In the group of SYP1 two syntaxins are already described. The tobacco NtSYR1 has a high homology with the *Arabidopsis* syntaxin AtSYP121 which is in the subgroup SYP12. NtSYR1 is involved in the signalling pathway of ABA in tobacco guard cells (Leymann *et al.*, 1999). KNOLLE (AtSYP111) belongs to the subgroup SYP11 and is involved specifically in the formation of the new cell plate during cytokinesis (Lukowitz *et al.*, 1996). The other 6 members of the SYP1 subclass have not yet been characterized.

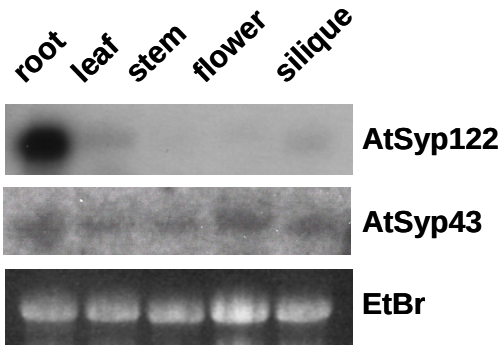

Figure 2 **Expression of *AtSyp122* and *AtSyp43* transcripts in different organs of *Arabidopsis***
Total RNA from the indicated organs was analysed by RNA blot hybridisation using probes for *AtSyp122* and *AtSyp43* as indicated in Figure 1. The exposure time of the *AtSyp43* blot was 5 times longer as for the blot of *AtSyp122*. Equal loading is shown by the agarose gel stained with ethidium bromide.

AtSYP43 belongs to the subclass SYP4 and shares high homology and identity with the two other members of this subclass (85% homology with AtSYP41 and 71% with AtSYP42; 79% identity with AtSYP41 and 57% with AtSYP42), see also Figure 1B. AtSYP41 and AtSYP42 are better known as AtTLG2a and b respectively. Originally TLG2a and b were identified and characterized in yeast by Holthuis *et al.*, (1998). The orthologs in *Arabidopsis thaliana* AtTLG2a and b are localized in different parts

of the TGN and form a complex with the Sec1-like/Munc-18 protein AtVps45 (Bassham *et al.*, 2000).

Expression of *AtSyp122* and *AtSyp43* in flowering *Arabidopsis* plants

The level of transcripts of *AtSyp122* and *AtSyp43* was analysed in different tissues of flowering plants and in roots grown in vitro (Figure 2). RNA hybridisation analysis was performed with a probe in a non conserved part of the sequence (see Figure 1A and 1B). *AtSyp122* was expressed only in roots grown in vitro and not in leaves, stems, flowers and siliques (Figure 2). *AtSyp43* transcripts were not detected in these tissues.

The expression of *AtSyp122* but not *AtSyp43* was induced after inoculation with pathogens

Three-week-old *Arabidopsis thaliana* accession Columbia plants were infected with the downy mildew *Peronospora parasitica* isolate Noco and Emwa. The isolate Noco forms a compatible interaction on accession Columbia in contrast to the isolate Emwa which forms an incompatible interaction (Holub et *al.*, 1994). In the compatible interaction the conidia of the oomycete were able to germinate and grow into the plant tissues, forming haustoria. After 5 – 7 days depending on the humidity, sporulation, the formation of conidiophores bearing the asexual conidia took place. In the incompatible interaction with the isolate

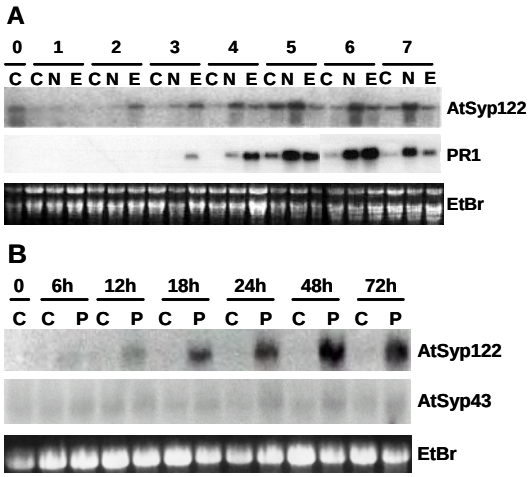

Figure 3 **Expression studies of *AtSyp122* and *AtSyp43* after inoculation with *Peronospora parasitica* and *Plectosporium tabacinum***
A) Time course of the expression of A*tSyp122* and *PR1* transcripts after inoculation with *Peronospora parasitica* isolates Noco and Emwa. Three week old plantlets were inoculated with *P. parasitica* isolate Noco (N) or Emwa (E) or water as control (C) and collected after different times in days as indicated. Total RNA was analysed by RNA blot hybridisation with the probes for *AtSyp122* and *PR1* as indicated in Figure 1.
B) Time course of the expression of *AtSyp122* and *AtSyp43* transcripts after inoculation with *Plectosporium tabacinum*. Three week old plantlets were inoculated with *P. tabacinum* (P) or water as a control (C) and harvested after different times as indicated. Total RNA was analysed by RNA blot hybridisation with the probes for *AtSyp122* and *AtSyp43* as indicated in Figure 1.
The RNA agarose gel stained with ethidium bromide is shown as a control for loading.

Emwa, the conidia were also able to germinate and to grow into the plant tissue but the hyphae growth was stopped after 3 – 4 days. There was no asexual sporulation. The expression of *AtSyp122* and of *PR-1* were analysed after inoculation with *P. parasitica* isolates Noco and Emwa (Figure 3). The level of *AtSyp122* transcripts increased after 3 - 4 days post inoculation (dpi) with *P.parasitica* isolate *Noco* and stayed high until the end of the time course (7d). In the incompatible interaction with isolate Emwa the level of *AtSyp122* transcripts increased earlier compared to the compatible interaction. It increased already at 2 - 3 dpi, had a maximum at 4 dpi and showed then a low level of expression until the end of the time course at 7 dpi. For comparison the level of expression of PR1 was also analysed (Fig 3A). Pathogenesis-related protein 1 is known to be induced after pathogen infection and is secreted to the cell wall (Uknes *et al.*, 1992). *PR1* was not expressed in control, non infected leaves and its expression was induced 3 dpi with *P. parasitica* isolate Emwa. It increased further after 4, 5 and 6 dpi and decreased after 7d. The expression of *PR1* increased 4 dpi after inoculation with *P. parasitica* isolate Noco and increased further after 5, 6 and 7 dpi.

The expression of *AtSyp122* and *AtSyp43* was analysed after infection with the virulent ascomycete pathogen *Plectosporium tabacinum*. The infection with *Plectosporium* leads to visible yellowing of the leaves after 2 days and after 3 - 4 days most of the leaves were macerated. The leaves were harvested 6, 12, 18, 24, 48 and 72h after inoculation. The expression of *AtSyp122* transcripts was induced after 18h post inoculation until the end of the time course. *AtSyp122* was not expressed in control, non infected leaves. *AtSyp43* expression was not induced after inoculation with *P. tabacinum* (Figure 3B).

The expression of *AtSyp122* and *AtSyp43* was highly induced in the knockout mutant *atsnap33*

A T-DNA insertion in the *AtSNAP33* gene causes loss of AtSNAP33 function, resulting in a lethal dwarf phenotype (Heese *et al.*, 2001). In the first 7 days of growth there was no visible difference between the wild type (Wassilewskija WS) and the *atsnap33* plantlets. When the cotyledons were fully developed (after 7 days) the formation of spontaneous and spreading lesions was observed. Once the cotyledons

were completely necrotised, the first true leaves showed also lesions and the *atsnap33* plantlets died after 3 weeks (Heese *et al.*, 2001). The mutant is not fertile and has to be maintained in the heterozygote stage and selected according to the phenotype at each generation. In the population used to maintain the mutant, 25 % of the seedlings are *atsnap33*, 50 % are heterozygotes (these show no visible phenotype) and 25 % are wild type. These seedlings can be distinguished one from the other by a PCR reaction using primers located in the T-DNA insert and in the neighbouring DNA region (Heese *et al.*, 2001). The expression of the syntaxins *KNOLLE (AtSyp111)*, *AtSyp122* and *AtSyp43* was analysed in 7 days old *atsnap33* and wild type plantlets grown on Petri plates containing ½ MS medium (Figure 4). *KNOLLE* was expressed in both type of plantlets. The expression of *AtSyp122* was high in the *atsnap33* plantlets and very low in the wild type. Interestingly, *AtSyp43* was not expressed in the wild type but was highly expressed in the *atsnap33* mutant.

Expression and purification of AtSYP122 and AtSYP43 in *E. coli*

In parallel to the expression studies of AtSyp122 and AtSyp43 we also were interested to purify AtSYP122 and AtSYP43 for *in vitro* binding assays with AtSNAP33 to confirm the yeast two hybrid results. As suggested by the manufacturer's instructions (Qiagen 3th edition, 1997 and Worrall, 1996) only the cytosolic part of the syntaxin i.e. without the transmembrane domain was fused with the Glutathione S-transferase (GST) as shown in Figure 5. The *E. coli* strain DH5α was transformed with the GST fusion vector alone and with the fusion protein GST::AtSYP122p, GST::AtSYP43p respectively which include both a site for cleavage by factor

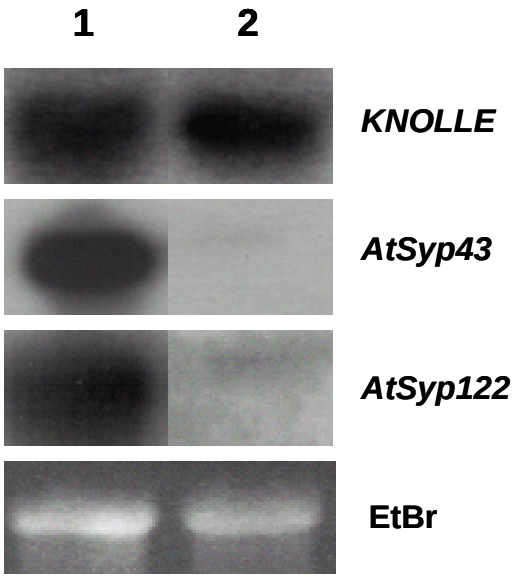

Figure 4 **Expression of *KNOLLE*, *AtSyp43* and *AtSyp122* transcripts in the knockout mutant *atsnap33***
Entire 7 day old plantlets grown on ½ MS medium were used for total RNA extraction. Total RNA was analysed by RNA blot hybridisation with the probes for *AtSyp122* and *AtSyp43* as indicated in Figure 1 The full length cDNA of *KNOLLE* was used as a probe. RNA from homozygote *atsnap33* mutants, line 1 and RNA from wild type (WS), line 2. The RNA agarose gel stained with ethidium bromide is shown as a control for loading.

Xa between the 2 proteins. The synthesis of the recombinant protein was induced by IPTG and after 1 – 4 h the bacteria were harvested and the total denaturated protein extract was separated by a SDS-PAGE and the gel was stained with Coomasie blue. Already 1h after the induction of the synthesis of the recombinant GST alone a new protein of 30 kDa appeared on the SDS-PAGE gel compared to a non induced protein extract which correspond to the calculated size of the GST (Figure 6A). The fusion protein GST::AtSYP122p has a calculated size of 60kDa. When the expression of this protein was induced a band of 56 kDa appeared on the SDS PAGE gel after 1 to 4h (Figure 6B). The fusion protein GST::AtSYP43p has a calculated size of 67 kDa. When the expression of this protein was induced a band of 58 kDa appeared on the SDS-PAGE gel after 2h (Figure 6C). The calculated sizes as well as the sizes determined of the different fusion proteins and the partial syntaxins after SDS-PAGE, are summarized in Table 1. The *E. coli* growth was not affected by the expression of the recombinant proteins and no other side effects were observed.

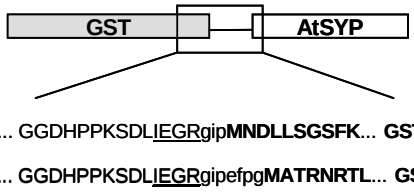

Figure 5 **Scheme and amino acid sequence of the fusion proteins GST::AtSYP122p and GST::AtSYP43p for recombinant protein expression in *E. coli***
The cytosolic part of AtSYP122 (aa 1 - 275) and AtSYP43 (aa 1 –307) were cloned in frame in the pGEX-5x-1 vector containing the sequence of GST followed by a cleavage site for the protease factor Xa. The upper part of the Figure represents a scheme of the fusion protein. A and B represent part of the amino acid sequence of the GST::AtSYP122p and GST::AtSYP43 protein. The sequence is represented in the following way: normal capital letters correspond to the C-terminal end of GST, underlined capital letters indicate the cleavage site for the factor Xa, the small letters stands for spacer amino acids produced by cloning and the bold capital letters correspond to the N-terminal part of the partial AtSYP122p respectively AtSYP43p.

Table 1: calculated and determined sizes of the recombinant proteins

recombinant protein	calculated size in kDa*	determined size from SDS-PAGE gel in kDa
GST	30	30
GST::AtSYP122p	60	56
AtSYP122	38	-
AtSYP122p	32	-
GST::AtSYP43p	67	58
AtSYP43	37	-
AtSYP43p	34	-

* analysed by Swiss Institute of Bioinformatics
www.expasy.org/tools/pi_tools.html
- no values available

The purification of the fusion protein GST::AtSYP122p from *E.coli* was performed by affinity on a glutathione Sepharose 4B column under native conditions. The eluate of the Sepharose column was separated on a SDS-PAGE gel. After Coomasie blue staining a band of 50 kDa corresponding to the fusion protein GST::AtSYP122 and some contaminating bands of smaller size were observed (see Figure 6D line 4). The eluate containing the fusion protein GST::AtSYP122p was incubated for different times with the protease factor Xa which cleaves the fusion protein between the GST and AtSYP122p. Two hours after the incubation of the fusion protein with the factor Xa the fusion protein of 50 kDa disappeared (Figure 6D line 5 – 8). The cleavage products were separated on the SDS-PAGE gel and 2 bands of 28 and 29 kDa appeared which makes the identification of the GST and AtSYP122p nearly impossible without further study (Figure 6D line 5 – 8). As size references the total lysate of the *E. coli* strain expressing the recombinant GST and the protease factor Xa were loaded on the same SDS-PAGE gel (Figure 6D line 2 and 3).

Most of the fusion protein GST::AtSYP43 expressed in *E. coli* was detected after extraction under native conditions in the insoluble part of the total lysate (data not shown). The soluble part of the fusion protein was used for the purification performed by affinity on a glutathione Sephasose 4B column. The eluate of the column was loaded on a SDS-PAGE and the gel was stained with Coomasie blue. There was not any proteins detected on the gel. Either the amount of the fusion protein GST::AtSYP43 was too low or the protein was not stable or premature degradation was responsible for the loss of the fusion protein GST::AtSYP43.

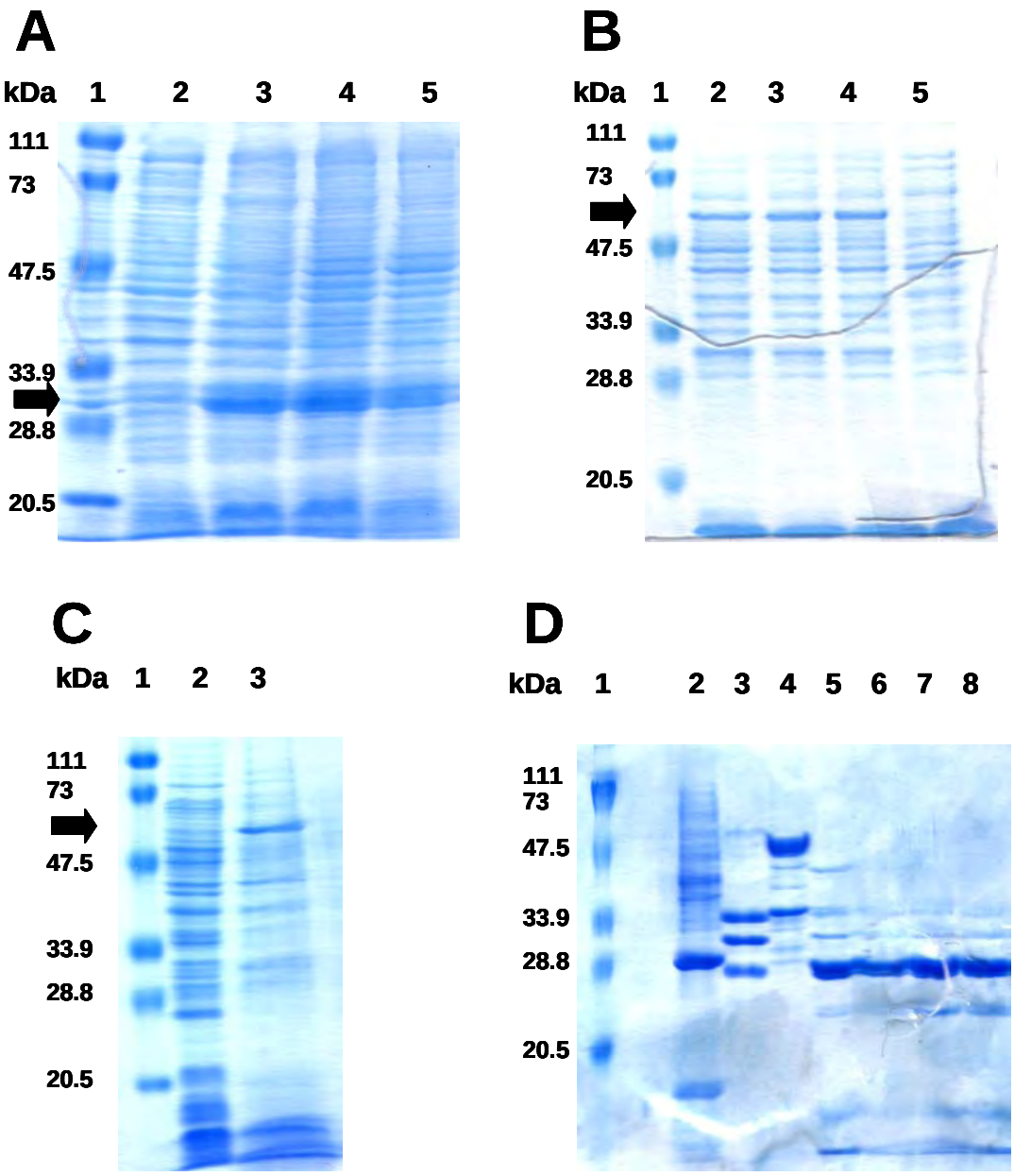

Figure 6 **A) Expression of the recombinant GST in *E. coli***
Expression of the GST::AtSYP122p and GST::AtSYP43p fusion protein in E. coli cell containing the recombinant construct grown in LB with ampicillin until a OD_{620} of 0.6 was reached. The expression of the recombinant protein was induced by IPTG. The bacteria were harvested before addition of IPTG and at different time points after addition of IPTG. Line 1 molecular weight standards, line 2 total denatured protein extract from *E. coli* cells before the addition of IPTG, line 3 – 5 total protein extract of cells harvested 1h, 2h and 4 hours respectively after addition of IPTG. The arrow indicates the expressed GST at 30 kDa.
B) **Expression of the GST::AtSYP122p fusion protein**
Line 1 molecular weight standards, line 2 – 4 total protein extract of cells harvested 1h, 2h and 4 hours respectively after addition of IPTG, line 5 total protein extract of no induced bacteria. The arrow indicates the recombinant GST::AtSYP122 at 56kDa.
C) **Expression of the GST::AtSYP43p fusion protein**
Line 1 molecular weight standards, line 2 total protein extract from from cells before addition of IPTG, line 3 total protein extract from cells 2 hours after addition of IPTG. The fusion protein is indicated with the arrow at 58 kDa.
D) **Cleavage products of the GST::AtSYP122p fusion protein after incubation with the protease factor Xa**
Line 1 molecular weight standards, line 2 total lysate of *E. coli* cells expressing GST, line 3 factor Xa, line 4 GST::AtSYP122p fusion protein purified on a glutathione Sepharose column, line 5 – 8 purified GST::AtSYP122p fusion protein incubated for 2, 4, 6, and 8h respectively with factor Xa.

DISCUSSION

In this study we show that the 2 syntaxins *AtSyp122* and *AtSyp43* have a very different expression pattern and that their expression is induced in different conditions. Both syntaxins were isolated by a yeast two hybrid screen with AtSNAP33, a homologue of the neuronal t-SNARE SNAP-25, as a bait.

The *AtSyp122* transcripts were constitutively expressed in roots but not in inflorescence stems, leaves, flowers and siliques. However *AtSyp122* transcripts were induced in leaves after inoculation with *Plectosporium tabacinum*. The expression of the *AtSyp122* transcripts were also after inoculation of leaves induced with *Peronospora parasitica* isolate Noco and Emwa which lead to a compatible and incompatible interaction, respectively, on *A. thaliana* accession Columbia plants. The level of *AtSyp122* transcripts was also increased by a mechanical stimulation such as touch (Studer, 2000 and Krattinger, 2001). Rubbing gently *Arabidopsis* leaves with ungloved fingers induced the expression of *AtSyp122* transcripts already after 15 minutes (Studer, 2000 and Krattinger, 2001). This expression was transient and *AtSyp122* transcript levels were back to control levels after 2h.

Based on sequences analysis AtSYP122 is a syntaxin which probably functions in the secretory pathway, forming a SNARE complex with other SNAREs. AtSYP122 has a transmembrane domain which indicate a possible localization to a membrane but the subcellular localization has to be determined.

The expression of *AtSyp122* was induced by pathogen and touch similarly to the expression of *AtSNAP33* (Wick, Xansel and Sticher unpublished result). It is possible that AtSNAP33 and AtSYP122 form a SNARE complex which is necessary for proper secretion of the proteins needed in these conditions, but this remains to be shown.

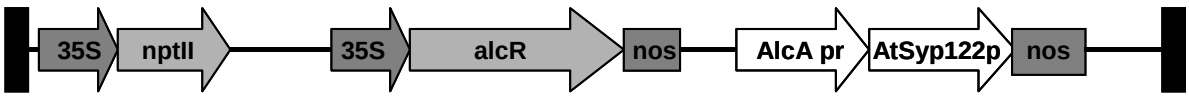

Figure 7 **T-DNA construct containing the ethanol inducible expression system for plants**
The black bars represent the left border and right border of the T-DNA. As a selective marker the nptII gene for kanamycin resistance was chosen. The 35S::alcR::nos cassette leads to a constitutive expression of the transcription factor alcR. The alcR transcription factor can bind to the AlcA promoter and induce the transcription of *AtSyp122p* only in the presence of ethanol. 35S, 35S promoter for constitutive expression in plants; nos, nos terminator

To study the function of AtSYP122, the protein without the transmembrane domain was expressed under the control of an ethanol inducible promoter system (Caddick *et al.*, 1998) in *Arabidopsis* cells. The partial AtSyp122p protein (aa 1 – 275) will stay in the cytosol and should bind to the SNARE partners. Thus AtSYP122p should poison the normal SNARE core complex formation. The phenotype of the transformed plant could give a hint on the function of AtSYP122. Leymann *et al.*, (1999) showed that the partial tobacco syntaxin NtSYR1 without the transmembrane domain prevents potassium and chloride ion channel response to ABA in guard cells.

The syntaxin AtSYP122p without the transmembrane domain was also fused to the ethanol inducible AlcA promoter (Figure 7) (Caddick *et al.*, 1998 and Salter *et al.*, 1998). The advantage of the inducible expression system is the prevention of possible lethality effects on the plants compared to the constitutive expression system. *Arabidopsis thaliana* accession Columbia plants were transformed with *AtSyp122p* by the *Agrobacterium* infection technique and the transgenic T1 lines were isolated but not yet characterized.
Another approach to learn more about the function of a protein is either overexpression or suppression of the expression of the protein of interest. For this reason the *AtSyp122* and *AtSyp43* cDNA were fused to the CaMV 35 promoter conferring constitutive expression in plants once in sense orientation for the overexpression and once in antisense orientation to suppress the translation of the corresponding syntaxin.

To test the interaction of AtSNAP33 and AtSYP122 an affinity assay was used. The binding of GST::AtSYP122p and His_6AtSNAP33 was tested on a glutathione Sepharose column to which GST::AtSYP122p was bound and His_6AtSNAP33 purified on a nickel column was added and after incubation the column was washed and the potential complex was eluted. With western blot analysis the presence of His_6AtSNAP33 was not detectable (L. Sticher, personal communication). This means that His_6AtSNAP33 and GST::AtSYP122 were not able to interact. It is not known if other compounds or proteins are necessary to form a stable complex. However, the fact that *AtSyp122* and *AtSNAP33* (Wick and Sticher, unpublished results) are coexpressed in roots and in leaves after pathogen inoculation is in agreement with a possible link between these two SNAREs.

The function of AtSYP43 is not better known. In contrast to *AtSyp122*, *AtSyp43* transcripts were not expressed in roots, inflorescence stems, leaves, flowers and siliques as analysed by RNA blot hybridisation. Neither pathogen inoculation nor mechanical stimulation (Studer, 2000) led to the induction of the expression of *AtSyp43*. The expression of the fusion protein GST::AtSYP43 in *E. coli* resulted in the appearance of a band at 58 kDa. However, this protein could not be purified on a glutathione Sepharose column. The protein could be aggregated in inclusion bodies since it was mainly detected in the insoluble part of the total protein lysate or the amount in the soluble part was too low for a successful purification.

Interestingly, the *AtSyp122* and *AtSyp43* transcripts were highly expressed in *atsnap33* plants which show no expression of AtSNAP33. The loss of function mutant *atsnap33* forms spontaneous spreading lesions 7 days after germination and finally dies after 3 – 4 weeks (Heese *et al.*, 2001). Why *AtSyp43* is expressed in these plants and not in all other conditions tested will be subject of further research. The syntaxins *AtSyp41* and *AtSyp42* from the same subfamily are localised at different parts of the TGN and may be required for different trafficking events (Bessham et *al.*, 2000). On the biological function of AtSYP43 we can only speculate. As a member of the SYP4 group, AtSYP43 could have similar function and localisation as AtSYP41 and AtSYP42.

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GENERAL DISCUSSION

The secretory pathway in plants is a complex endomembrane system. It transports proteins to the extracellular space or to the vacuole by budding of vesicles from a donor membrane and fusion with an acceptor membrane. The fusion of the appropriate vesicles with the correct target membrane is of outmost importance, otherwise the cell will lose its compartmentalisation and collapse. Soluble *N*-ethylmaleimide-sensitive factor adaptor proteins receptors (SNAREs) and their associated factors were described to be responsible for these fusions steps.
In this work AtSNAP33, a homologue of the SNAP-25 t-SNARE, from *Arabidopsis thaliana* was functionally characterized.
In chapter one AtSNAP33 was isolated as an interactor of the cytokinesis-specific syntaxin KNOLLE by a yeast two-hybrid screen. AtSNAP33 is an ubiquitously expressed membrane-associated protein that is localized at the plasma membrane and during cell division colocalize with KNOLLE at the forming cell plate. A T-DNA insertion in the AtSNAP33 genes caused loss of AtSNAP33 function resulting in a lethal dwarf phenotype. Atsnap33 mutants developed large spreading necrotic lesions first on the cotyledons and later on the whole tissue except the roots.
AtSNAP33 is localized at the plasma membrane without having a transmembrane domain. Since all SNAP-25 homologues have no unique mechanism to be attached at the membrane, each member as to be tested how it is linked to the membrane. A conserved cysteine cluster has been shown to be palmitoylated and responsible for membrane association of SNAP-25 (Veit *et al*., 1996). SNAP-29 a mammalian homologue of SNAP-25 as well as Sec9p and Spo20p both yeast homologues of SNAP-25 do not have such a cluster (Steegermaier *et al*., 1998). The fact that AtSNAP33 is only solubilized in the presence of SDS a lipid anchor would be a possible mechanism for membrane association since a single cysteine at position 119 is present in AtSNAP33.

In chapter two the focus was specially on the expression pattern of AtSNAP33. After attack by different pathogens, *Arabidopsis* respond with a whole set of defence reactions as well as the induction of AtSNAP33. It was shown that salicylic acid is able but not necessary to induce AtSNAP33 in leaves inoculated with *Pseudomonas*

syringae, whereas the systemic induction of AtSNAP33 after pathogen attack is SA dependent. Also other stresses like UV, wounding and mechanical stimulations such as touch and wind induce the expression of AtSNAP33.

The signal perception and transduction pathway after different applied stresses leading to the expression of AtSNAP33 is unknown. Further experiments are needed to gain more detailed information about the regulation of AtSNAP33. Mutants in different signal pathways known to be involved during pathogenesis were screened for AtSNAP33 expression after touch. There were no differences in the expression of AtSNAP33 after touch in the *Arabidopsis* mutant *etr1*, *jar1*, *eds5* and *sid2* compared to the wild type plants (Page, 2000; Studer, 2000; Kratinger, 2001). This leads to the conclusion that these mutants are not interfering with the signalling pathway leading to the expression of AtSNAP33 after touch.

In parallel *Arabidopsis* plants overexpressing AtSNAP33 were generated and analysed (Xavier Gansel and Liliane Sticher unpublished results). There was no visible changes observed in the phenotype of these plants. These plants were inoculated with pathogens to test if the plant have altered response in resistance by inoculation with *P. parasitica.* No differences were observed (Beeler, 2001).

Chapter three is the continuation of the detail analysis of the ‘knockout’ mutant *atsnap33*. The loss of AtSNAP33 lead to a dwarf phenotype. The spontaneous formation of spreading lesions leads to the dead of the mutant after 3 - 4 week after germination. A reduction of secretion activity was measured in the *atsnap33* mutant, compared to the wild type plant. To support this observation a subcellular analysis focused on the organelles involved in the secretory pathway could reveal details about the inhibited secretion. The *atsnap33* mutants have such a dwarf phenotype that analysis of processes are difficult. Another way to study the function of AtSNAP33 is the expression of AtSNAP33 without the last 10 amino acids. It was shown by Criado *et al*. (1999) that the lack of the last few amino acids of SNAP-25 reduces the secretion activity in chromaffin cells. A partial AtSNAP33 expressed under the control of an inducible promoter would probably bind to the corresponding SNARE partners without being able to form a functional SNARE core complex. It is not excluded that the same phenotype would be observed after induction of the partial AtSNAP33. The advantage of this system is the possibility of a focused analysis of the processes involved before the phenotype is expressed.

In the last chapter of this work two novel syntaxins AtSYP122 and AtSYP43 were isolated by a yeast two hybrid screen with AtSNAP33 as a bait (Xavier Gansel and Liliane Sticher unpublished results). AtSYP122 expressed in the roots of *Arabidopsis* and induced by pathogen attack promise to be a interesting potentially interacting partner of AtSNAP33. Its function and localisation has still to be investigated. AtSYP43 was not expressed in roots, leaves, inflorescent stem, flowers and siliques. Both AtSYP122 and AtSYP43 were highly expressed in the 'knockout' mutant *atsnap33*. The reasons of this particular expression pattern is unknown. Moreover the function and the localisation of AtSYP43 and AtSYP122 are not known. *Arabidopsis thaliana* has two homologues of AtSNAP33, AtSNAP29 and AtSNAP30. Their characterization is in the very beginning and would be very interesting in the understanding of their function and their relation to AtSNAP33.

Two independent yeast two hybrid screen revealed only potential partners of AtSNAP33 from the same protein family the syntaxins. To find the third component of the SNARE core complex, i.e synaptobrevin like proteins, another strategy has to be applied. It is not excluded that for the complex formation supplementary compounds are needed which were absent in the yeast two hybrid.

The fact that AtSNAP33 interacts potentially with several syntaxin like KNOLLE (Heese et al., 2001), NtSYr1 (Kargul et al., 2001) as well as AtSYP122 and AtSYP43 give the impression that AtSNAP33 interacts in different SNARE core complexes. This hypothesis is also supported by the fact that the number of syntaxins, distributed in all compartment involved in the secretory pathway is bigger than the number of SNAP-25 like SNARE proteins.

The functional characterization of AtSNAP33 added new knowledge on the understanding of the secretion in plants. But based on these results new interesting aspects are opened which drive the research in this field further.

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