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Review

Annexins — calcium- and membrane-binding proteins in the plant kingdom

Potential role in nodulation and mycorrhization in *Medicago truncatula*

Tanuja Talukdar[1], Karolina Maria Gorecka[1], Fernanda de Carvalho-Niebel[2], J. Allan Downie[3], Julie Cullimore[2] and Slawomir Pikula[1]✉

[1]*Department of Biochemistry, Nencki Institute of Experimental Biology, Warszawa, Poland;* [2]*Laboratoire des Interactions Plantes-Microorganismes, INRA-CNRS, Castanet-Tolosan, France; Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Norwich, UK*

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Annexins belong to a family of multi-functional membrane- and Ca^{2+}-binding proteins. The characteristic feature of these proteins is that they can bind membrane phospholipids in a reversible, Ca^{2+}-dependent manner. While animal annexins have been known for a long time and are fairly well characterized, their plant counterparts were discovered only in 1989, in tomato, and have not been thoroughly studied yet. In the present review, we discuss the available information about plant annexins with special emphasis on biochemical and functional properties of some of them. In addition, we propose a link between annexins and symbiosis and Nod factor signal transduction in the legume plant, *Medicago truncatula*. A specific calcium response, calcium spiking, is an essential component of the Nod factor signal transduction pathway in legume plants. The potential role of annexins in the generation and propagation of this calcium signal is considered in this review. *M. truncatula* annexin 1 (MtAnn1) is a typical member of the plant annexin family, structurally similar to other members of the family. Expression of the *MtAnn1* gene is specifically induced during symbiotic associations with both *Sinorhizobium meliloti* and the mycorrhizal fungus *Glomus intraradices*. Furthermore, it has been reported that the MtAnn1 protein is preferentially localized at the nuclear periphery of rhizobial-activated cortical cells, suggesting a possible role of this annexin in the calcium response signal elicited by symbiotic signals from rhizobia and mycorrhizal fungi.

Keywords: Nod factors, plant annexins, *Medicago truncatula*

INTRODUCTION

Calcium (Ca^{2+}) signaling and its maintenance of cellular homeostasis is one of the most important signal transduction pathways now in the vast field of plant science. Among the Ca^{2+} binding proteins potentially involved, in this review we focus on plant annexins. The special feature of these Ca^{2+}-binding proteins is that they can behave as cytosolic, peripheral or integral membrane proteins in a Ca^{2+} sensitive and insensitive manner. They could play a role in the determination and orientation of membrane dynamics in endo- and exocytosis. In the first section of this review, we describe plant annexins including their structures and a wide range of functions, while in the second section we formulate our hypothesis about the potential role of annexin 1 from *Medicago truncatula* (MtAnn1) in the Nod factor (NF) signal transduction pathway.

✉Corresponding author: Slawomir Pikula, Department of Biochemistry, Nencki Institute of Experimental Biology, L. Pasteura 3, 02-093 Warszawa, Poland; e-mail: s.pikula@nencki.gov.pl

Abbreviations used: Ann, plant annexin; Anx, vertebrate annexin; Hac, hair curling; Had, hair deformation; ITs, infection threads; LRR-RLK, leucine-rich-repeat receptor-like-kinase; LysM-RLKs, lysin motif-receptor-like-kinases; NFs, Nod factors; RLK, receptor-like-kinase; NFP, Nod factor perception; Rip1, Rhizobium-induced peroxidase 1; ENOD11, ENOD40, early nodulin 11 and 40; ROS, reactive oxygen species.

PLANT ANNEXINS

General introduction about plant annexins

Annexins are ubiquitous, soluble proteins capable of Ca^{2+}-dependent and Ca^{2+}-independent binding of phospholipids of endomembranes and of plasma membrane. Annexins are a diverse and multifunctional family in animal cells, where they are involved in a wide variety of essential cellular processes. Plant annexins constitute a smaller and less diverse family of proteins, which are grouped in a distinct monophyletic group. They have been found in all monocot and dicot plants tested to date and are represented by seven, nine or ten genes in Arabidopsis, rice and Medigaco, respectively (de Carvalho-Niebel *et al.*, 2002; Mortimer *et al.*, 2008). Molecular sequence analysis revealed that the distinct annexin subfamilies in plants and protists in case of *Giardia lamblia* (Anx21-Gla) diverged prior to animal annexins and thus are derived from a common ancestor (Morgan *et al.*, 1997; 1999; 2004). Plant annexins have been found to represent at least 0.1% of the total protein in those cases studied so far (Clark & Roux, 1995). The first evidence that higher plants contain annexin-like protein was reported in tomato (Boustead *et al.*, 1989). Different members of the plant annexin family of proteins are capable of binding to F-actin, hydrolyzing ATP and GTP and acting as peroxidases or as cation channels. These *in vitro* properties together with their specific subcellular localizations and expression patterns have implicated these proteins in the regulation of plant growth and signaling during both abiotic stress (Mortimer *et al.*, 2008) and nodulation (de Carvalho-Niebel *et al.*, 1998; 2002). Their role in these processes may be mediated through maintenance of homeostasis of cytosolic free calcium (Ca^{2+}) and reactive oxygen species (ROS) in the cellular environment (reviewed by Gerke & Moss, 2002; Hofmann, 2004; Gerke *et al.*, 2005).

The annexin protein family is a multigene, multifunctional family of soluble proteins with a vast taxonomic distribution. The annexin sequences have been described in more than sixty five species including plants, fungi, protists, higher vertebrates and recently a prokaryote. The name "annexin" is derived from the Greek word "annex" meaning "bring/hold together/aggregate" to describe the principal property of this protein family as binding to or holding together certain biological structures, particularly membranes (reviewed by Gerke & Moss, 2002).

Plant annexin structure

The molecular mass of plant annexins is in the range of 32–42 kDa. Annexins have an evolutionary conserved overall structure composed of a C-terminal conserved core containing a four-fold repeat (I–IV) of 70 amino-acid residues, the so-called "annexin repeat", showing homology in linear sequence alignments among different species and do not contain an EF hand Ca^{2+}-binding site. Each repeat of the C-terminal core consists of a four-helix bundle (A, B, D, E) and a fifth helix (C) almost perpendicular to those bundles. Plant annexins have in general a short N-terminal region (preceding the first structural repeat), distinct from their animal counterparts that possess a variable (in length and size) N-terminal region. In animal annexins the N-terminal region is the site for secondary modifications including phosphorylation, nitrosylation, S-glutathiolation and N-myristoylation which participate in the regulation of several distinct signaling pathways (reviewed by: Gerke & Moss, 2002; Gerke *et al.*, 2005). A nearly full-length plant annexin cDNA was obtained from *Medicago sativa* (Pirck *et al.*, 1994). This sequence appears to lack the N-terminal 10–40 amino-acids but contains the characteristic four 70- to 75-amino-acid structural repeats and shows 37% sequence identity with a human annexin sequence. The first three-dimensional structure of a member of the plant annexin family and the crystal structure of annexins from *Capsicum annuum* (Ann24Ca) showed that the short N-terminal region interacts with the annexin core which suggests that some regulatory function of this region is conserved in plant annexins (Hofmann *et al.*, 2000). This crystal structure of Ann24Ca is shown in Fig. 1.

In the case of plant annexins only the first and fourth repeats contain the canonical calcium (Ca^{2+}) binding sites provided by the characteristic endonexin fold sequence (K-G-X-G-T-{38}-D/E) (Geisow *et al.*, 1986), involved in Ca^{2+} binding (Mortimer *et al.*, 2008). The annexin domain forms a highly α-helical and tightly packed slightly-curved disk which is formed by the C-terminal (core) domain of annexins, of which there exists only one per annexin (excluding annexin A6). The more convex side, facing towards the membrane when annexins remain bound peripherally with membrane phospholipids, comprises the 17 amino-acid endonexin fold region with its characteristic KGhGTDEXXLIpILApR motif (h = hydrophobic residue, X = any residue, p = polar residue), containing the type II Ca^{2+} binding sequence of GXGTD (de Carvalho-Niebel *et al.*, 1998; Clark *et al.*, 2001; Mortimer *et al.*, 2008). The more concave side, which remains away from the membrane, interacts with the NH_2 terminal domain and also possibly with other cytoplasmic Ca^{2+} binding partners (reviewed by: Gerke & Moss, 2002; Gerke *et al.*, 2005; Hofmann *et al.*, 2000; 2003). The crystal structures of plant annexins have been determined in the case of *Capsicum annuum*, *Gossypium hirsutum*

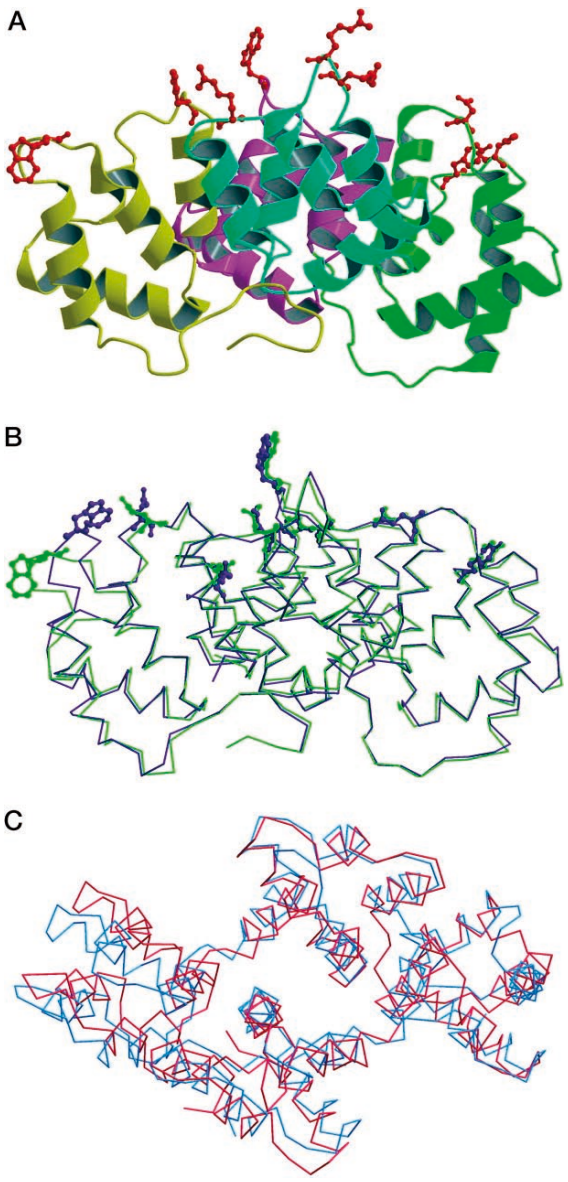

Figure 1. Crystal structure of plant annexins, particularly annexin 24 (Ca32).
A. The different domains of this annexin are depicted as follows: domain 1: yellow; domain 2: magenta; domain 3: green; domain 4: blue. **B**. Superposition of molecule 1 (green) and molecule 2 (purple) of annexin 24 (Ca32). **C**. Superposition of molecule 1 of annexin 24 (Ca32) (red) and AnxA5 (blue) are viewed from the membrane binding side. Reprinted from Hofmann *et al.*, (2000) *J Biol Chem* **275**: 8072–8082, with permission from the Authors and Publisher.

and *Arabidopsis thaliana* (Hofmann *et al*., 2000; 2003). In Fig. 1 the crystal structure of annexin 24 from *C. annuum* (Anx24-Ca32) has been described along with its highly exposed residues within the membrane binding loops and superposition of two annexin molecules. A recent study to investigate which calcium binding sites are utilized by plant annexins found that three calcium ions in type II and type III binding sites in repeats I and IV are located in the crystal structure of AnnGh1. The conformation found in the IDE loop region would allow binding of a fourth Ca^{2+} ion (Hu *et al*., 2008). In Fig. 2 the multiple sequence alignment among MtAnn1, MtAnn2, AtAnn1 and human annexin shows the presence of the first and fourth Ca^{2+} binding site, phospholipid binding site, F-actin binding site and putative peroxidase domain in MtAnn1 and MtAnn2 primary sequence, but absence of GTP binding site. The sequence similarity between MtAnn1 and AtAnn1 gene is 80%. This latter protein has been shown to act as an ion chnnel (Gorecka *et al*., 2007).

Subcellular localizations of plant annexins

The majority of plant annexin proteins are found in the cytosol, but some can be found associated with the plasma membrane, endomembranes or the nuclear envelope (Blackbourn *et al*., 1992; Clark *et al*., 1992; 1994; Thonat *et al*., 1997; de Carvalho-Niebel *et al*., 2002). In *M. truncatula,* an annexin has been found in a root plasma membrane detergent-insoluble microdomain or the "lipid rafts" fraction (Lefebvre *et al*., 2007). Annexins can also be associated with membrane vesicles or Golgi, implicating them in membrane trafficking processes. Certain members have also been found in the nuclei or to relocalize in the cell in response to certain stimuli (Thonat *et al*., 1997; Clark *et al*., 1998; 2000; Mortimer *et al*., 2008). The subcellular localization of a particular annexin may depend on cellular pH, cytoplasmic Ca^{2+} concentrations, tissue type and plant species.

The annexin functions in plants: identification of functional motifs

Interaction with acidic phospholipids

The Ca^{2+}-dependent binding to negatively charged acidic membrane phospholipids has been a landmark characteristic feature of the annexin protein family due to its evolutionary-conserved structural motifs (Geisow *et al*., 1986). In the presence of micromolar cytoplasmic Ca^{2+} concentrations, annexins bind reversibly to negatively charged membrane phospholipids: phosphatidylserine, phosphatidylinositol and phosphatidic acid and the Ca^{2+} requirement for binding can be reduced by acidic pH and is reversed by Ca^{2+} chelators (Blackbourn *et al*., 1991; Balasubramanian *et al*., 2001). Annexins can act as cytosolic, peripheral and even integral membrane proteins depending on cellular pH, cytoplasmic Ca^{2+} concentration, membrane oxidation, lipid composition and voltage (reviewed by: Gerke & Moss, 2002; Ladokhin & Haigler, 2005). The Ca^{2+} requirements for plant annexin binding to phospholipids extend

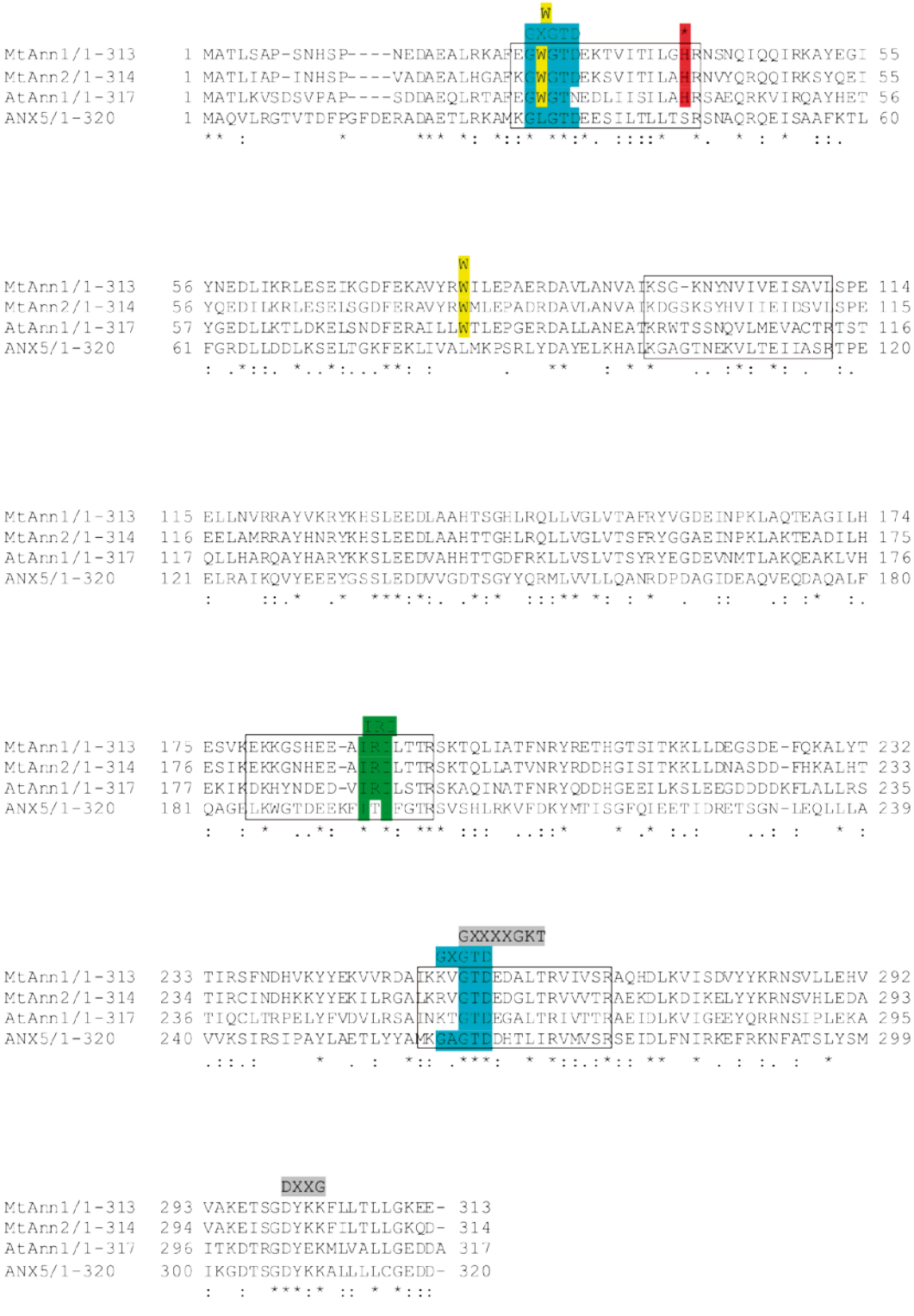

Figure 2. Alignment of primary structures of MtAnn1 and MtAnn2 with annexin 1 from *A. thaliana* and human annexin, by CLUSTAL W 1.83 multiple sequence alignment.

The sequences highlighted are as follows: red, for peroxidase activity the conserved His residue; box, 17 amino-acid endonexin fold region with its characteristic KGhGTDEXXLIpILApR motif (type II Ca^{2+} binding sequences are shown with marked GXGTD in blue); green, F-actin binding sequence marked as IRI; ash, GTP binding motif is marked as GXXXXGKT and DXXG; yellow, phospholipid binding site marked as W. Sequence data in this alignment can be found in the EMBL/GenBank data libraries under accession numbers gb:CAA75308 (MtAnn1), gb: C20129 (MtAnn2), gb:NP174810 (AtAnn1), gb:AAB60648 (human AnxA5).

over a broad range from below 10^{-7} to above 10^{-4} M (Clark & Roux, 1995). The binding of the annexin AnnAt1 (from *Arabidopsis thaliana*) to asolectin liposomes is largely prevented by a chelating agent, but is promoted in the presence of micromolar levels of Ca^{2+} (Gorecka *et al.*, 2005). The required Ca^{2+} concentrations for AnnAt1-liposome binding is three orders of magnitude lower in comparison to required Ca^{2+} concentration for mammalian annexins under *in vitro* conditions (Gorecka *et al.*, 2005). There is also evidence for significant binding of AnnAt1 to liposomes in the absence of Ca^{2+} (Gorecka *et al.*, 2005). In the case of plants, hydrophobic interactions are involved in annexin interactions with membrane phospholipids. The bell pepper (*Capsicum annuum)* AnnCa32 interacts with membrane phospholipids and this involves hydrogen bonding of several amino-acid residues to the phospholipid head group and glycerol backbone. Although annexins bind to membrane phospholipids in a Ca^{2+}-dependent manner at neutral pH 7.0–7.4, this binding can also occur in the absence of Ca^{2+}. According to a recent report, about 20% of annexins (cotton AnnGh1 and bell pepper AnnCa32) remain bound to lipid vesicles in the absence of Ca^{2+} at neutral pH (Dabitz *et al.*, 2005). These reports indicate the existence of populations of annexins interacting with membrane phospholipids in Ca^{2+}-dependent and Ca^{2+}-independent manner (Mortimer *et al.*, 2008). Interestingly, the annexins from *Zea mays* coleoptiles, p35, p33, and p23, do not contain the conserved domain responsible for Ca^{2+} binding and they do not associate with detergent-insoluble cytoskeletal proteins or with F-actin, although p33 and p35 share the common annexin characteristic of binding to membrane lipids. The hypothesis that plant annexins mediate exocytotic events is supported by the fact that p23, p33 and p35 bind to these secretory vesicles in a Ca^{2+}-dependent manner (Blackbourn *et al.*, 1991; 1992). The partial protein sequence analysis of plant annexins from *Lycopersicon esculentum,* p35.5 and p34, revealed sequence similarity to the 70-amino-acid-residue repeat region found in all annexins sequenced (Smallwood *et al.*, 1990; 1992). The first complete sequences for the widely reported doublet of plant annexins are p33 and p35 from the root tip forms of maize (Battey *et al.*, 1996).

Possible role of annexins in intracellular calcium homeostasis

The resting cytoplasmic Ca^{2+} concentration in plant cells is in the range of 100–200 nM (Clark & Roux, 1995). In a micromolar range of cytoplasmic Ca^{2+} concentration annexins interact with membrane phospholipids in a Ca^{2+}-dependent manner. Different signals like light, hormones, gravity, touch, cold, drought, wind, oxidative stress and functional elicitors have been reported to increase the cytoplasmic Ca^{2+} concentrations. The different Ca^{2+} sensors, Ca^{2+}-binding proteins and Ca^{2+} pumps in the cells maintain this nanomolar range of cytoplasmic resting Ca^{2+} concentrations. The sensors include (I) calmodulin (CdM), (II) other calmodulin-like Ca^{2+}-binding proteins with EF hand motifs, (III) Ca^{2+}-regulated protein kinases and (IV) other Ca^{2+}-binding proteins without EF hand motifs including annexins (reviewed by Reddy, 2001). It is known that annexins participate in Ca^{2+} homeostasis within animal cells (Gerke *et al.*, 2005), but this has not been experimentally demonstrated for any plant annexin.

Annexins in intracellular ion homeostasis

Ca^{2+} binding is a characteristic feature of annexin proteins. Studies on animal annexins describe these proteins as capable of sensing and regulating free cytosolic Ca^{2+}. Animal annexins can also act as Ca^{2+} permeable ion channels themselves. This ability was first demonstrated in the case of bovine AnxA7 (Pollard & Rojas, 1988). Annexin channel activity depends on ATP, GTP, cAMP, hydrogen peroxide and pH. The ability of plant annexins to form or regulate Ca^{2+} channels in plasma and endomembranes enable signal transduction and amplification (Kovacs *et al.*, 1998; White *et al.*, 2002). The putative pore region of the human AnxA5 channel contains two salt bridges (Asp92-Arg117 and Glu112-Arg271) which regulate selectivity for Ca^{2+} and channel opening in response to voltage (Liemann *et al.*, 1996). These two salt bridges are well conserved in the case of plant annexins. Some plant annexins may act as plasma membrane Ca^{2+}-permeable channels for Ca^{2+} entry into the cell at very negative (hyperpolarized) membrane voltages (White *et al.*, 2002). For example, the bell pepper annexin AnnCa32 mediates passive Ca^{2+} flux in Fura-2-loaded vesicles supporting the concept of this annexin playing a role as a channel (Hofmann *et al.*, 2000). The maize annexins AnnZm33/35 contain putative salt bridges and partially purified protein preparations of these two annexins show hyperpolarisation-activated Ca^{2+} permeable channels in planar lipid bilayers (Nichols, 2005) and AnnAt1 from *A. thaliana* can form low specificity cation-permeable channels at low pH in lipid bilayers (Gorecka *et al.*, 2007).

Peroxidase activity of plant annexins

Plant cells contain suites of proteins regulating $[Ca^{2+}]_{cyt}$ and reactive oxygen species (ROS)-mediated signaling. ROS and $[Ca^{2+}]_{cyt}$ appear to act sequentially in some networks. Thus proteins like annexins with the ability to regulate these two key network components could be significant control points. Oxidative stress results from the imbalance between the formation of free radicals and their neutralization by antioxidants. Various processes disrupt this

balance by increasing the formation of free radicals in relation to the available antioxidants. Free radical formation and the effect of these toxic molecules on cell function (which can result in cell death) are collectively called "oxidative stress". As a defense mechanism, plants are able to neutralize the effects of overproduction of reduced oxygen species (ROS) during oxidative stress by activating their protective mechanisms, through the induction of antioxidant enzymes such as catalase, glutathione transferase, superoxide dismutase and peroxidases. Hofmann *et al.* (2003) identified in the crystal structure of cotton annexin AnnGh1 the S3 cluster formed by two adjacent cysteine residues and a nearby methionine residue. It may play a role in transfer of electrons to an oxidizing molecule which could be reactive oxygen species (ROS). ROS generation has been shown to be involved in plant growth and development and sometimes also in the control of cytoplasmic Ca^{2+} concentration (Gapper & Dolan, 2006). The channel forming annexins, such as AnnAt1, are also candidates for the ROS-activated channels identified in plant cells (Foreman *et al.*, 2003). AnnAt1 has been shown to have the ability to play a role in sensing oxidative stress as it contains an unusual S3 cluster formed by two cysteines and a methionine (as identified first in cotton annexin AnxGh1 — Hofmann *et al.* (2003) which could act as a possible redox state sensor (Gorecka *et al.*, 2005).

The first annexin domain of some plant annexins shows strong sequence similarity with the heme binding motif of plant peroxidases, as typified by horse radish peroxidase and it contains a conserved histidine residue H40 (Gidrol *et al.*, 1996; Clark *et al.*, 2001; Gorecka *et al.*, 2005). In *Escherichia coli*, AnnAt1 carrying a mutation in the H40 residue showed reduced peroxidase activity, thus showing the potential functional relevance of H40 in this activity (Gorecka *et al.*, 2005). AnnAt1 from *Arabidopsis thaliana* rescued the Δ*oxyR* mutant of *E. coli* from H_2O_2 and oxidative stresses (Gidrol *et al.*, 1996; Kush and Sabapathy, 2001). Plant annexins could play a role as peroxidases, as shown by recent work on recombinant AnnAt1 of *Arabidopsis thaliana* (Gorecka *et al.*, 2005). Post-translational modifications are probably necessary for this peroxidase activity of AnnAt1 because the enzyme showed higher activity when expressed in *N. benthamiana* than in *E. coli* and the activity was decreased by dephosphorylation. The theoretical molecular mass and isoelectric point of AnnAt1 is 36 kDa and pI 5.2. Two-dimensional electrophoresis identified two microsomal spots were found for AnnAt1: 40 kDa, pI 5.2 and 40 kDa, pI 5.3 (Lee *et al.*, 2004), suggesting post-translational modification. Gorecka *et al.* (2005) found that AnnAt1 oligomerised at pH of 4.5 but the functional significance of this is not known. It is notable that annexin nitrosylation has been found in *Arabidopsis* (Lindermayr *et al.*, 2005).

F-actin binding of annexins

It has been suggested that actin binding requires the presence of an IRI (isoleucine-arginine-isoleucine)-motif in the annexin (Clark *et al.*, 1995). The actin-binding capacity of annexins differs between species. Tomato and *Mimosa* annexins both undergo Ca^{2+}-dependent F-actin binding *in vitro* involving interactions of cytoskeleton and cellular membranes (Calvert *et al.*, 1996; Hoshino *et al.*, 2004). *Mimosa* annexin organizes F-actin into thick bundles in the presence of Ca^{2+} *in vitro* (Hoshino *et al.*, 2004). Annexins from zucchini bind to zucchini-derived F-actin (Hu *et al.*, 2000). Cotton, bell pepper and maize annexins show no affinity for actin in the presence or absence of Ca^{2+} (Blackbourn *et al.*, 1992; Delmer & Potikha, 1997; Hoshino *et al.*, 2004). According to the multiple sequence alignment in Fig. 2 we also found the presence of F-actin binding domain in the AnnMt1 sequence.

ATPase and GTPase activity of annexins

Some plant annexins can bind purine nucleotides and hydrolyze them (maize, McClung *et al.*, 1994; tomato, Calvert *et al.*, 1996; Lim *et al.*, 1998; cotton, Shin & Brown, 1999). This nucleotide binding and hydrolysis could depend on the presence of a Walker A motif (GXXXXGKT/S) and a GTP-binding motif typical of the GTPase superfamily (DXXG) (Clark *et al.*, 2001). *Arabidopsis* annexins, AnnAt2 and AnnAt7 contain such motifs in the fourth repeat (Clark *et al.*, 2001). But *M. truncatula*, MtAnn1 does not contain this motif as shown in Fig. 2. GTP in millimolar range promotes the voltage-dependent ion-channel activity of animal annexins in planar lipid membranes (Kirilenko *et al.*, 2002).

Other functions

In addition to the functions described above, plant annexins also play roles in secretion, exocytosis, cell expansion and plant growth and development, adaptive signaling including touch, cold, salinity and in light response, nyctinastic movement, gravitropism, response to stress stimuli including drought, cold and salt stress and pathogens. The studies of the pattern of plant annexin gene expression in different plants including *Lysopersicon esculentum*, *Hordeum vulgare* and *Solanum tuberosum* revealed the highest levels of abundance of annexins in the root, lower levels in the stem and young leaves, and no expression in mature leaves (Smallwood *et al.*, 1992). A strawberry annexin clone, Rj4, has been detected as a fruit-specific annexin (Wilkinson *et al.*, 1995). A 42 kDa annexin-like protein, VCaB42, is associated with plant vacuoles and correlates with the expan-

sion of tobacco cells leading to a possible role for this protein in vacuole biogenesis (Seals *et al.*, 1994; Seals & Randall, 1997). The cotton fibre annexin bind to and inhibit the activity of a partially purified cotton fibre callose synthase thus indicating a possible function of plant annexins is to modulate the activity and/or localization of callose synthase (Andrawis *et al.*, 1993). The study on annexin localization in plant cells described that p35 from *P. sativum* is diffusely distributed throughout the nuclear structure indicating a possible role for plant annexins in nuclear functions (Clark *et al.*, 1998). Annexin-mediated secretion during gravitropism and distribution of annexins in the cells of gravistimulated pea plumules has been shown (Clark *et al.*, 2000). In *Arabidopsis* tissue specific analysis of different genes showed distinct expression profiles, suggesting specialized function in the plant family. To date, the studies on plant annexins have focused on annexin structure and *in vitro* functions. These previous studies have shown that annexins are multifunctional with various possible *in vivo* roles.

THE POTENTIAL ROLE OF ANNEXIN 1 FROM *MEDICAGO TRUNCATULA* (MtAnn1) IN NODULATION AND MYCORRIZATION — A HYPOTHESIS

Symbiosis and Nod perception

Symbiotic interactions are established between legume roots and soil bacteria collectively known as rhizobia, which include the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium, Mesorhizobium* and *Sinorhizobium.* These interactions involve two important steps: (I) a controlled infection process (root penetration by the bacteria) and (II) the formation of the specialized root organs called nodules, in which the bacteria fix atmospheric nitrogen into ammonium.

A signal exchange initiates the symbiosis; it involves flavonoids (or other inducers) from legume hosts which induce bacterial *nod* genes, whose products synthesize lipo-chitooligosaccharide (LCO) signals, Nod factors (NFs), which are usually essential for the symbiosis (Dénarié *et al.*, 1996). Attachment of NF-producing bacteria to the root hairs of host plants leads to morphological changes (Dénarié & Cullimore, 1993) such as root hair deformation (Had) and root hair curling (Hac). Biological responses are induced by very low Nod factor concentrations (10^{-9} to 10^{-12} M) (Catoira *et al.*, 2000). Bacterial infection generally occurs through root hair cells that curl around rhizobia, entrapping the attached bacteria. Tubular structures called infection threads (ITs) are initiated within the curled root hairs, and the bacteria grow along the extending root hair which grows down through root cortical tissues. In temperate legumes such as *Medicago truncatula*, inner cortical cells undergo mitotic activity and develop into a nodule primordium, where bacteria differentiate into bacteroids which fix nitrogen (Brewin *et al.*, 1998) and the infecting rhizobia bud off from the infection threads and are released into the newly divided plant cells. A meristem is formed and this sustains subsequent growth and differentiation of a nodule structure. The *M. truncatula* nodule is referred to as being indeterminate because it contains a persistent apical meristem. This meristem region in a mature nodule is referred to as zone I, and there are also other zones called the invasion zone II (where infection of the plant cells takes place), the amyloplast-rich interzone II–III, the fixation zone III (where rhizobia differentiate into nitrogen-fixing bacteroids) and the senescence zone IV (where bacterial cells degenerate) (reviewed by Gage & Margolin, 2000). Other legumes including beans and *Lotus japonicus* produce 'determinate' nodules in which the meristematic activity is transient. The establishment of *M. truncatula* together with *L. japonicus* as model organisms has allowed the identification of the first host components involved in NF signal transduction and perception (Geurts *et al.*, 2005). The knowledge about the role of Ca^{2+} binding protein annexins in inducing Ca^{2+} signaling during initiation of nodulation and mycorrhization in legume plants is limited. So in the 2nd part of our review we will focus on our knowledge about these proteins in *M. truncatula.* This species, a close alfalfa relative, has been established as a model plant for legume biology because it possesses a number of interesting characteristics for both molecular and classical genetics including diploidy and autogamous fertilization, a small genome (500–600 Mbp/1C), a rapid reproductive cycle, a high level of biodiversity, a number of cultivars and a well characterized nitrogen-fixing symbiont, *Sinorhizobium meliloti* (Thoquet *et al.*, 2002).

Nod factor signaling pathway and NF-induced Ca^{2+} spiking

NF signals are likely to be perceived by highly specific Lysin Motif-Receptor-Like Kinases (LysM-RLKs) (Radutoui *et al.*, 2007), which, in *M. truncatula,* include NFP (Amor *et al.*, 2003) and LYK3 (Smit *et al.*, 2007). According to the Nod factor signaling pathway functioning downstream of these LysM RLKs are DMI1, a putative cation channel, and DMI2, another receptor-like kinase with leucine-rich repeat domains (LRR), which are both required for the generation of Nod factor-induced calcium spiking (for review see Oldroyd & Downie, 2004). Calcium spiking is one of the important events in the

Nod factor signaling pathway. It originates in the nuclear membrane and then propagates outwards as a wave (Shaw & Long, 2003). Induction of Ca^{2+} spiking upon NF perception is dependent upon both DMI1 and DMI2, whereas NF-induced influx of extracellular Ca^{2+} is independent of the *DMI* genes (Shaw & Long, 2003). The nuclear envelope and the nuclear-associated endoplasmic reticulum are both potential internal stores for the calcium released during Nod factor-induced calcium spiking (Peiter *et al.*, 2007). Recently DMI1 has been shown to be localized along the nuclear membrane (Riely *et al.*, 2007) and has been suggested to regulate calcium channels associated with the nuclear periphery to generate NF-induced calcium spiking (Peiter *et al.*, 2007). The precise mechanism of induction of calcium spiking is not known but it seems likely that it requires a secondary message. Inhibitors of phospholipases C and D inhibit calcium spiking and early nodulation gene induction and this points towards a possible role for phospholipids generating a secondary signal that in turn activates calcium spiking. Inside the nucleus, calcium calmodulin-dependent kinase (CCaMK) encoded by *DMI3* is required for transduction of the calcium spiking signal leading to activation of specific early nodulin gene expression *via* specific GRAS and ERF transcription factors (for a review see Oldroyd & Downie, 2008).

Common signaling pathway in nodulation and mycorrhization

It is notable that several of the early Nod-factor signaling genes including *DMI1, DMI2* and *DMI3* are also required for the establishment of a symbiosis with arbuscular mycorrhizal fungi and mycorrhizal fungal association can also induce calcium spiking (Kosuta *et al.*, 2008). This demonstrates that there is a common signaling pathway for the establishment of both symbioses and it is thought that the nodulation pathway evolved from the much more ancient mycorrhizal signaling pathway (for a review see Kistner & Parniske, 2002).

A new NF-induced gene encoding annexin 1 MtAnn1

Based on the observation that Nod factor-induced *AnnMt1* gene encodes a protein which is structurally homologous to the other members of calcium binding annexin protein family (de Carvalho-Niebel *et al.*, 1998; 2002), it has been hypothesized that this AnnMt1 plays an important role in NF-induced calcium spiking (for review see Oldroyd & Downie, 2008). Experimental evidence supporting the involvement of this protein in the cellular response to calcium signal generation remains scant. Further studies are necessary for the investigation of biochemical structural and functional characterizations of AnnMt1 in order to prove the above hypothesis experimentally.

Annexins in *M. truncatula* and their putative roles in Nod factor signal transduction

The model legume *M. truncatula* is likely to contain at least ten different annexins including the symbiosis-induced *MtAnn1* and *MtAnn2* genes (de Carvalho-Niebel *et al.*, 1998; 2002; Manthey *et al.*, 2004). MtAnn1 and MtAnn2 share 75% sequence identity and are quite divergent from the stress-related *M. sativa* annexin gene *AnnMs2* (35% identity with other plant annexins) (Kovacs *et al.*, 1998). This divergence suggests specialized functions within this gene family. Both *MtAnn1* and *MtAnn2* show enhanced expression in rhizobial-inoculated roots and nodules. *MtAnn1* was first described as a new early nodulin gene up-regulated in root tissues responding to Nod factors (de Carvalho-Niebel *et al.*, 1998), while the *MtAnn2* gene was identified by a transcriptomic approach as being activated during both arbuscular mycorrhizal and rhizobial symbioses (de Carvalho-Niebel *et al.*, 2002; Manthey *et al.*, 2004). In mature nodules, *MtAnn2* is expressed in peripheral nodule tissues restricted to the vasculature, whereas *MtAnn1* expression is associated with differentiating cells in the distal part of the nodule tissues in invasion zone II that will be later infected by rhizobia. The analyses of p*MtAnn-GUS* fusions in transgenic *M. truncatula* roots revealed *MtAnn2* expression to be associated with dividing cells of nodule primordia, while *MtAnn1* transcription is either directly activated by NFs or during the symbiotic association of roots with *S. meliloti*, both in inner and outer root tissues. It thus appears that the symbiotic MtAnn1 and MtAnn2 annexins are associated with distinct symbiotic events occurring during early symbiotic stages related either to bacterial penetration or nodule organogenesis and mycorrhizal infection of the root cortex (Manthey *et al.*, 2004).

In particular, the symbiotically induced MtAnn2 has been reported to be expressed during both arbuscular mycorrhizal and rhizobial symbioses. More recently analysis of *M. truncatula* root proteins identified MtAnn1 and one related isoform to be increased in abundance in response to inoculation with the mycorrhizal fungus *Glomus intraradices* (Amiour *et al.*, 2006). The up-regulation is associated with early stages of arbuscular mycorrhizal (AM) appressorium formation which is in line with the early activation of MtAnn1 during *S. meliloti* preinfection and infection symbiotic stages. This may point to a function of MtAnn1 during a common early regula-

tory pathway shared between the two symbioses (de Carvalho-Niebel *et al.*, 1998; Amiour *et al.*, 2006).

Biochemical characterization has for the moment only been reported for the MtAnn1 protein. It shows calcium-dependent binding to acidic phospholipids, which is typical of the annexin family. Confocal studies demonstrated that a MtAnn1-GFP fusion protein localizes to the cytoplasm but preferentially accumulates at the nuclear periphery of *S. meliloti*-activated outer cortical cells (de Carvalho-Niebel *et al.*, 2002). Nod factor-induced calcium spiking associated with the nuclear envelope is a well characterized early response in the NF signaling pathway and a similar response has recently been shown to be involved in early mycorrhization signaling pathway (Kosuta *et al.*, 2008). The fact that MtAnn1 is induced in both symbioses points to the possibility that it could play a role in the early signaling pathway preceding early nodulin and mycorrhizal-induced gene activation in roots.

Potential role of MtAnn1 in the production of phospholipid-based secondary messengers

In animal systems, the induction of Ca^{2+} spiking is observed through phospholipid signaling that is driven by phospholipase C. Reports on pharmacological inhibitors indicate that phospholipase C is involved in NF-induced Ca^{2+} spiking and expression of NF-induced genes (Engstrom *et al.*, 2002). Biochemical evidence indicates that both phospholipase C and phospholipase D are activated by NFs (den Hartog *et al.*, 2001; 2003). Taken together, these studies indicate a possible combined role for phospholipase C and phospholipase D in the generation of phospholipid-based signals, which could induce Ca^{2+} spiking. A secondary messenger, phospholipid, might link NF perception at the plasma membrane with Ca^{2+} changes in the nucleus (for review see Oldroyd & Downie, 2008).

Potential role of MtAnn1 in the induction of Ca^{2+} spiking

The nuclear membrane-localized protein, DMI1, required for NF-induced Ca^{2+} spiking (Riely *et al.*, 2007) functions as a potassium channel (Charpentier *et al.*, 2008) and has been proposed to regulate calcium channels in both yeast and plants (Edwards *et al.* 2007; Peiter *et al.*, 2007; Charpentier *et al.*, 2008; Oldroyd & Downie, 2008). This potassium channel might be the target of the secondary messenger and the resultant changes in potassium may drive membrane hyperpolarization which could activate a voltage-gated calcium channel leading to Ca^{2+} spiking. As Nod factor-induced nuclear membrane MtAnn1 belongs to the Ca^{2+} binding annexin protein family, it may play a role in the maintenance of Ca^{2+} homeostasis and in induction of Ca^{2+} spiking.

As shown in Fig. 3, we propose a model that integrates MtAnn1 as a potential actor in the NF signal transduction. In view of the known properties of annexins, there are two aspects of the nodulation (and by analogy mycorrhizal) signaling pathway that could be affected. (I) Since annexins can bind phospholipids it is possible that they could influence the production of secondary messengers, phospholipids, that are predicted to be produced as a result of phospholipase activity. (II) Alternatively, because plant annexins may act as Ca^{2+}-permeable channels in the plasma membrane, it is possible that the symbiosis-induced MtAnn1 annexin could participate in the nuclear-associated calcium spiking if MtAnn1 acts at the nuclear membrane. It is possible for example that MtAnn1 could influence the activity of the nuclear membrane-localized DMI, which is proposed to regulate ER-associated calcium channels to generate calcium spiking (Edwards *et al.*, 2007; Peiter *et al.*, 2007). Because the major expression of *MtAnn1* is found in root cortical cells, a second NF-signaling event could exist, involving specific calcium changes in these cells prior to bacterial penetration. Miwa *et*

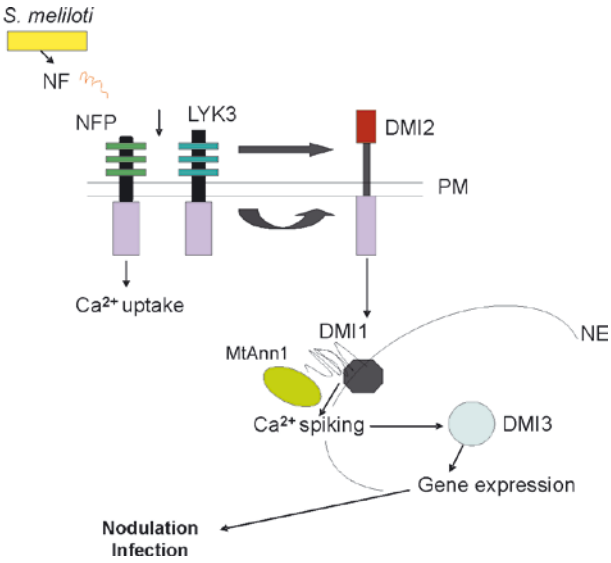

Figure 3. Symbiotic interactions and Nod factor signal transduction pathways.
The symbiotic interactions between the legume plant *Medicago truncatula* and the soil bacterium *Sinorhizobium meliloti* are established by the release of Nod factors (NF) from the bacterium. These signals are perceived and transduced by, the plasma-membrane (PM) located LysM-RLKs, NFP and LYK3 and the leucine-rich-repeat receptor-like-kinase DMI2. NF-induced Ca^{2+} changes are central components of this signal transduction pathway. Calcium spiking also requires DMI1, which has been recently localised to the nuclear envelope (NE). A similar location of MtAnn1 suggests a role of this annexin in the calcium response signal. Calcium spiking is transduced *via* DMI3, to elicit gene expression leading to nodulation and infection.

al. (2006) proposed that increased levels of Nod factors accumulating in infection threads could induce the observed calcium influx across the cell membrane, and that this could lead to initiation of infection. Thus a plant annexin MtAnn1 could influence this calcium influx as well.

CONCLUSIONS AND FUTURE PERSPECTIVES

This brief review summarizes the vast literature on functional characterization of annexins from different plant species including the legume *M. truncatula*. Annexins represent members of membrane phospholipid and calcium binding proteins in the cellular environment of both plants and animals. In the references mentioned in this review the structural, biochemical and functional properties of annexins from different plant species have been reported. These properties include interactions with membrane phospholipids, Ca^{2+} and ROS homeostasis, peroxidase activity, cation channel activity, actin binding, ATPase and GTPase activity, functioning in plant growth and development and response to abiotic stress. From the review of the literature, annexins could also play an important role in Nod factor signaling in the establishment of the legume-rhizobia symbiosis. To understand this role detailed investigations are necessary on the biochemical and functional characterizations of MtAnn1 and its role in activating calcium signal generation and maintaining homeostasis.

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