Ion transport mediated by the Slow Vacuolar channel in plant cell vacuoles: biophysical properties and modulation by endogenous molecules

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Abbreviations:

AA  Arachidonic Acid
ABC transporter  ATP-Binding Cassette transporter
At  Arabidopsis thaliana
CICR  Calcium Activated Calcium Release
DHA  DocosaHexaenoic Acid
DMSO  DiMethyl SulfOxide
ER  Endoplasmic Reticulum
Fou2  Fatty acid oxygenation up regulated 2
AtGt  Arabidopsis thaliana Glucosyl transferase
GST  Glutathione S Transferase
MATE  Multidrug And Toxic Efflux
MRP  Multidrug Resistance associated Protein
NAADP  Nicotinic Acid Adenine Dinucleotide Phosphate
Nar  Naringenin
PUFAs  PolyUnsaturated Fatty Acids
QCT  Quercetin
SV channel  Slow Vacuolar channel
TPC1  Two Pore Channel 1
tt  transparent testa
YFP  Yellow Fluorescent Protein
Abstract in English

The Slow Vacuolar channel, ubiquitous in plant cells, has been investigated for almost 25 years; nevertheless its role remains still elusive. In search of compounds that are able to change its properties providing useful information for its physiological role, we investigated the modulation of the SV channel by the flavonoid Naringenin (Nar) using the patch-clamp technique. Experiments were conducted on vacuoles obtained from carrot roots and from mesophyll cells of Arabidopsis thaliana leaves. Firstly, the presence of non-glycosylated Nar in the cytoplasm was verified by localization studies, then patch-clamp experiments in the excised patch configuration revealed that Nar inhibits the SV channel in a reversible dose-dependent manner, shifting the threshold of activation of the SV currents towards more positive membrane potentials. Cytosolic Nar did not change the amplitude of the single channel conductance but decreased the mean open time of the channel. We also demonstrated that Nar inhibits equally well the permeation of both K+ and Ca2+ through the SV channel. Flavonoids sharing structural similarities with Nar, induced a comparable inhibition of the SV currents. Inside-out patch experiments performed in vacuoles from Arabidopsis thaliana mesophyll cells and carrot roots revealed that Nar inhibits the SV channel also when added at the luminal side. Naringin, the glycosylated form of Nar, was unable to change the channel activity when added at both sides of the membrane.

Finally, in accordance with a higher hydrophobicity of the flavonoids at acidic pHs, Nar inhibition increases decreasing the pH of the cytosolic bath solution. All these evidences point to the possibility that flavonoid interaction with the SV channel is mediated by the membrane phospholipids.

To further investigate this possibility we studied the interaction of polyunsaturated fatty acids (PUFAs) with the SV channel in vacuoles from carrot roots. Similarly to Nar, both Arachidonic Acid (AA) and Docosahexaenoic Acid (DHA) added at the cytosolic side induced a dose-dependent reversible decrease in the SV channel activity. Also AA shifted the SV activation to more positive voltages without changing the amplitude of the single channel conductance.

These studies suggest that important endogenous molecules, such as flavonoids and PUFAS, are able to change the properties of the slow vacuolar channel, possibly through a lipid mediated interaction. This biophysical characterization opens new perspectives to investigate the properties of this enigmatic channel in different plant species and tissues.
Il canale Slow Vacuolar (SV), un canale ubiquitario in sistemi vegetali, è studiato da circa 25 anni ma il suo ruolo fisiologico rimane tuttora elusivo. Alla ricerca di molecole che potrebbero essere in grado di modularne le proprietà fornendo informazioni utili alla comprensione del suo ruolo fisiologico, mediante metodologia di “patch-clamp abbiamo studiato le modificazioni indotte da Naringenina (Nar) su detto canale in vacuoli estratti dalla radice di carota o dal mesofillo della foglia di Arabidopsis thaliana. La presenza nel citoplasma di Naringenina, un flavonoide non-glicosilato, è stata preventivamente verificata mediante studi di localizzazione. Successivamente esperimenti di patch-clamp nella configurazione “excised-out” hanno dimostrato che Nar inibisce il canale SV in modo reversibile e dipendente dalla concentrazione di flavonoide mentre la soglia di attivazione del canale viene spostata verso potenziali più positivi. L’aggiunta di Nar alla soluzione citosolica non cambia l’ampiezza di singolo canale ma diminuisce aumenta il tempo di vita media del canale in stato aperto. Abbiamo anche dimostrato che Naringenina inibisce allo stesso modo sia il trasporto di K⁺ che di Ca²⁺ attraverso il canale SV. Inoltre i flavonoidi con struttura simile a quella di Naringenina inducono inibizioni confrontabili delle correnti SV. Esperimenti effettuati in configurazione “inside out” su vacuoli ottenuti da Arabidopsis e da carota hanno dimostrato che Nar inibisce il canale SV anche quando essa viene aggiunta dal lato vacuolare. Invece Naringin, la forma glicosilata della Naringenina, non è in grado di cambiare l’attività del canale sia che essa venga aggiunta al lato citosolico che al lato vacuolare. Infine, in accordo con l’aumento di idrofobicità dei flavonoidi utilizzati a pH acidi, l’inibizione delle correnti SV mediate da Nar aumenta diminuendo il pH della soluzione citosolica. Tutte queste evidenze suggeriscono che l’interazione tra flavonoidi e canale SV possa essere mediata dai lipidi di membrana. Per sviluppare ulteriormente questa ipotesi di lavoro abbiamo studiato l’interazione di alcuni acidi grassi polinsaturi (PUFAs) con il canale SV del vacuolo di carota. In maniera del tutto simile a quanto accade all’aggiunta di Nar, sia l’acido Arachidonico (AA) che l’acido Docoesaenoico (DHA), aggiunti alla soluzione citosolica, inducono una diminuzione reversibile e dose-dipendente dell’attività del canale. Come la Naringenina, anche AA sposta il potenziale di attivazione delle correnti SV verso valori di potenziali più positivi senza cambiare l’ampiezza di singolo canale. Questi studi suggeriscono che alcune importanti molecole endogenee, quali i flavonoidi ed i PUFAs, sono in grado di modificare le proprietà del canale Slow Vacuolar, probabilmente attraverso un’interazione mediata dai lipidi di membrana. Questa caratterizzazione biofisica apre nuove prospettive per lo studio delle proprietà di questo enigmatico canale in specie vegetali ed in tessuti diversi.
State of the art and Objectives
Chapter 1.1. The Plant Vacuole

Most plant cells contain liquid-filled membrane-bound organelles called vacuoles. A vacuolar membrane called tonoplast surrounds the plant vacuole. In mature plant cells, the vacuole can occupy from 30% to 90% of the cell volume. Classification of the vacuoles is based on the nature of their soluble proteins and by the class of aquaporins present on the tonoplast.

![Schematic representation of an idealized plant cell with a large central vacuole.](image)

**Fig. 1.1** Schematic representation of an idealized plant cell with a large central vacuole.

Mature plant cells may contain two large vacuoles with different functions (Martinoia et al. 2007). For example (1) the central vacuole plays a role in the maintenance of turgor pressure, in cell homeostasis and in the storage of metabolic compounds, (2) defence or signal compounds may be stored in the vacuoles of some cell types (Marty 1999), (3) proteins are stored in the vacuoles of reserve tissues of fruits and seeds (Herman and Larkins 1999), (4) vacuoles can be involved in programmed cell death (Paris et al. 1996). Transport across the tonoplast is essential for cell homeostasis and to maintain the turgor pressure which is fundamental in regulation of cell expansion and growth (Maeshima 2001). As the central vacuole occupies most of the space in the cell, it stores all the
essential compounds, minerals, nutrients, metabolites and different ions required for cell homeostasis. Different systems of ion transport were identified in the vacuolar membrane, which allow the ions to selectively permeate according to their electrochemical gradients (channels) or at the expenses of other energy sources (active transporters).

Chapter 1.2. **Ion transport in plant vacuoles**

1.2.1 **Vacuolar Transporters**

In plant cells compounds are transported across the tonoplast for storage and some compounds present in the cytoplasm may reach up to 7 fold higher concentrations in the vacuole. For example, the pH in the lumen can reach values up to 3 units whereas in the cytoplasm it remains around 7 units. It is obvious to note that proton (H⁺) fluxes are required to maintain this pH difference and two proton pumps, namely a V-ATPase and a V-PPase responsible for this phenomenon (Sze et al. 1999; Maeshima 2000), are present in all types of vacuoles (Hedrich et al. 1989). By pumping the protons into the lumen they not only remove them from the cytosol but also create a voltage difference across the tonoplast of about -30 mV (cytosol minus vacuolar side). The electrochemical pH gradient and the voltage difference are used by channels (see channels section below) and

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**Fig. 1.2. Schematic representation of the vacuolar transporters (Shiratake and Martinoia 2007).**

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secondary active transporters to transfer electrically charged ions, in accordance or against their chemical or electrochemical potential difference, respectively. It is shown that a potential difference of -30 mV would allow ~5 fold vacuolar accumulation of monovalent anions, (such as chloride or nitrate) and ~10 fold accumulation of divalent anions (such as sulfate) relative to the cytosol concentrations (Martinoia et al. 2007).

**Vacuolar H⁺-ATPase**

V-ATPase uses the energy of ATP hydrolysis to pump the protons across a variety of biological membranes. The existence of a V-ATPase was reported way back in 1962 (Kirshner 1962), in plants in 1973 (Rungie and Wiskich 1973). More recently, it was found out to be universally present in all the membranes of different internal acidic organelles in eukaryotic cells (Martinoia et al. 2007).

![Vacuolar H⁺-ATPase](image)

**Fig. 1.3.** Schematic representation of a vacuolar H⁺-ATPase. The V-ATPase complex is composed of a peripheral domain (V₁), which interacts with ATP, ADP and inorganic phosphate. The V₁ domain is a hexamer of A and B subunits. The V₀ domain is composed of a ring of proteolipid subunits (c, c' and c''), adjacent to subunits a and e. This complex mediates the transport of H⁺. The V₁ and V₀ domains are connected by a central stalk, composed of subunits D and F of V₁ and subunit d of V₀, and multiple peripheral stalks, composed of subunits C, E, G, H, and a. From Carraro-Lacroix et al (2009).

Their multi subunit composition was revealed by purification of vacuolar ATPases from animals, plants and fungi. They are made up of 14 different polypeptides, which assemble as two major ring structures (**Fig. 1.3**): (1) a peripheral V₁ complex that interacts with ATP, ADP and inorganic phosphate, and (2) an integral membrane V₀ complex that
mediates the transport of H⁺. In eukaryotic V-type H⁺ ATPases, the V₁ complex is invariably present in the cytoplasm. In plants V-ATPase activity is transcriptionally regulated and it has been demonstrated that post translantional modifications by a WNK (With No K = lysine) kinase are involved in this regulation (Hong-Hermesdorf et al. 2006). V-ATPase activity is inhibited by bafilomycin (Bowman et al. 1988). For a critical review on the structure, function and physiological role of V-ATPases see Beyenbach and Wieczorek (2006).

**Vacuolar H⁺-pyrophosphatase**

The V-PPase proton pump uses PPI as its energy source, the compound that is synthesized in many metabolic reactions. This type of pump is found in archaebacteria, protozoa, algae, photosynthetic bacteria and plants but not in fungi or mammals (Martinoia et al. 2007). The molecular identity of a V-PPase from *Arabidopsis thaliana* plant was reported by Kieber and Signer (1991). It consists of a single polypeptide with a molecular mass of about 80 kDa. It has been suggested that the V-PPase activity is regulated by sodium (Rea and Poole 1985), calcium (Ca²⁺) (Rea et al. 1992) and magnesium (Leigh et al. 1992; Baykov et al. 1993) from the cytosolic side. Two isoforms of this pump are found in plants: a potassium-dependent type which is moderately sensitive to calcium and a potassium-independent form that is extremely calcium sensitive (Belogurov and Lahti 2002). Both types of isoforms require magnesium as a cofactor. V-PPase activity is essential for maintaining the acidity of the large central vacuole in plant cells (Maeshima 2000). Generally, its activity is high in young tissues, but in grape berries it is expressed even in mature plant cells (Terrier et al. 1998). For reviews on V-PPases see Baltscheffsky et al. (1999), Maeshima (2000) and Gaxiola et al. (2007).

**Calcium ATPase**

Calcium can constitute up to 5% of the dry weight of a plant (Broadley et al. 2004). The large central vacuole is the most important free Ca²⁺ storage organelle in plant cells (Martinoia et al. 2007). The free Ca²⁺ concentration inside the vacuole is around ~ 1000 – fold higher than in the surrounding cytosol (Bush 1995), where the free Ca²⁺ concentrations are around 100 nM-200 nM at rest (Wheeler and Brownlee 2008). Ca²⁺ is one of the most important second messengers in plant cells; an increase in intracellular
calcium is believed to be a major pathway in the plant stress response. The huge Ca\(^{2+}\) concentration differences (between the store and surrounding cytosol) are the basis for the function of Ca\(^{2+}\) as second messenger (Pottosin and Schonknecht 2007). Ca\(^{2+}\)-ATPases and Ca\(^{2+}/H^+\) antiporters perform the active Ca\(^{2+}\) transport into the vacuole (Sarafian et al. 1992). The Ca\(^{2+}\)-ATPases are one of the best-characterized ion transport ATPases in the organisms. The plant Ca\(^{2+}\)-ATPases are divided into two groups: type IIA and IIB. In *Arabidopsis thaliana* four isoforms of IIA Ca\(^{2+}\)-ATPase (AtECA) (Geisler et al. 2000) and six isoforms of type IIB Ca\(^{2+}\) ATPase (AtACA) (Sze et al. 2000) have been cloned.

**Ca\(^{2+}/H^+\) Antiporter**

The Ca\(^{2+}/H^+\) antiporter is driven by the transmembrane pH gradient generated by the V-ATPase and V-PPase. This antiporter was reported to be present in various plant species (Schumaker and Sze 1986; Chanson and Pilet 1987; Berkelman et al. 1994). The first Ca\(^{2+}/H^+\) antiporter from *Arabidopsis thaliana* was cloned in 1996 (Hirschi et al. 1996). It has 11 transmembrane domains and a highly acidic motif between the sixth and the seventh transmembrane domains.

**Na\(^+\)/H\(^+\) Antiporter**

The Na\(^+\)/H\(^+\) antiporter can export sodium (Na\(^+\)) from the cytosol to extracellular space as well as into the vacuole using proton gradients (Blumwald et al. 2000). The first Na\(^+\)/H\(^+\) exchanger identified in plants was *Arabidopsis thaliana* AtNHX1 protein (Gaxiola et al. 1999). These are proteins of about 550 residues in length and have 10–12 transmembrane domains with a hydrophilic C-terminal tail in the luminal side (Yamaguchi et al. 2003). It has been shown that the Na\(^+\)/H\(^+\) antiporter plays a major role in salt tolerance (Blumwald and Poole 1985). For review see Pardo et al. (2006).

**ATP-Binding Cassette Transporters**

The ABC transporter super family is the largest protein-family known, and its members are capable of a numerous transport functions (Henikoff et al. 1997). The ABC transporters are reported in several species ranging from archaeabacteria to humans (Higgins 1992; Volkl et al. 1996). They are capable of transporting alkaloids, lipids,
peptides, steroids, sugars, inorganic anions, and heavy metal chelates with the exception of inorganic anions; MgATP directly energizes the transport of all of these substances (Rea et al. 1998). All ABC transporters are constituted of one or two copies each of two basic structural elements: a hydrophobic, integral transmembrane domain containing four to six transmembrane helices, and a cytoplasmically oriented ATP-binding domain. In plants herbicide detoxification is performed by Multidrug Resistance associated Proteins (MRP) that belongs to the ABC transporter family. From *Arabidopsis thaliana* plants four MRP genes have been cloned: AtMRP1 (Lu et al. 1997), AtMRP2 (Lu et al. 1998), AtMRP3 (Tommasini et al. 1998) and AtMRP4 (Sanchez-Fernandez et al. 1998). For review on plant ATP-binding transports see Rea (2007).

**Multidrug And Toxic compound Extrusion Transporters**

The MATE transporter was first reported from bacteria (Morita et al. 1998) and its homologs were later reported from plants and animals. *Arabidopsis thaliana* plant contains 56 MATE transporter genes in its genome (Yazaki et al. 2008). MATE transporters are involved in the transport of plant secondary metabolites. They consist of 400-700 amino acids with 9-12 transmembrane domains (Yazaki et al. 2008). In *Arabidopsis thaliana* plants MATE Transporter TT12 acts as a vacuolar flavonoid/H⁺-Antiporter (Marinova et al. 2007b). For review on MATE transporters see Yazaki et al. (2008).

**Aquaporins**

Aquaporins are small (23-31 kDa) membrane proteins containing six transmembrane domains that belong to the family of Major Intrinsic Proteins (MIPs). They are present in microbes, animals and plants. They not only transport water but also small neutral solutes and gases (Tyerman et al. 2002). On the basis of sequence homology plant aquaporin family can be subdivided into four groups: the Plasma membrane Intrinsic Proteins (PIP), Tonoplast Intrinsic Proteins (TIP), Nodulin-26–like Intrinsic membrane Proteins (NIPs) and Small basic Intrinsic Proteins (SIPs). In *Arabidopsis thaliana* plant 10 homologs of TIPs have been reported (Quigley et al. 2002). For classification and functions of plant aquaporins see Maurel et al. (2008).
1.2.2 Vacuolar Channels
The electrochemical potential existing across the vacuolar membrane is very important for any ion to be transported via channel mediated transport (Allen and Sanders 1997). Differently from transporters, ion channels can conduct a large number of ions/sec with high conductances ranging from 1 pS (pico siemens) to several thousand pS. Vacuolar channels can be broadly divided into two major groups: anion channels and cation channels. Though less studied anion channels have equal importance in the homeostasis of the cell.

Anion Channels
The most abundant anions in plants are malate and nitrate. Malate plays a major role in plant carbon metabolism and has many functions in plant cells, in charge balancing in the vacuole, in the carboxylate and glyoxylate cycle, in CO₂ temporary storage in C4 plants, and in stomata and pulvini movements. In many of these processes, malate is accumulated into the vacuole (Martinoia and Ratajcsak 1997). The malate channel is the most studied anion channel in plant vacuoles, and it has been found in all plants studied till now (Hafke et al. 2003; Hurth et al. 2005). These channels are selective for malate as well as for other organic acids such as fumarate but typically impermeable to citrate. The accumulation of malate mediated by channels is driven by the electrochemical gradient across the tonoplast. This gradient is generated by malate concentrations, and by the transtonoplastic potential created by the proton pumps. The molecular identity of malate channels has been revealed by the identification of the AtALMT9 protein (Arabidopsis thaliana Aluminium-activated Malate Transporter) (Kovermann et al. 2007).

The other major anion in plants is nitrate. Nitrate is an important source of nitrogen for plants and most plants devote a significant portion of their energy for its uptake and assimilation. Nitrate serves both as a nutrient and as a signal transducer and has profound effects on plant metabolism and growth. The cytoplasmic nitrate has two major destinies: (1) to be assimilated in organic compounds, and (2) to be accumulated into the vacuole. The unassimilated nitrate is transported and stored in the vacuole up to concentration values of 50-80 mM (Martinoia and Wiemken 1981). The accumulated nitrate is then redistributed to cytoplasm under starvation.
It has been shown that in *Arabidopsis thaliana* plants the members of Cloride Channel (CLC) family, specifically AtCLCa is involved in nitrate homeostasis (De Angeli et al. 2006; De Angeli et al. 2009). Actually AtCLCa is not a channel (as initially hypothesized) but a transporter that uses the proton gradient to actively accumulate nitrate into the vacuole.

**Cation channels**

Potassium (K\(^+\)) is the most important and abundant cation present in plant cells. Its concentration may reach up to 10% of the plant dry weight. Potassium plays a role in cellular processes like cell homeostasis, stomatal and leaf movements. Plants take up potassium from the soil and plant cells accumulate K\(^+\) to regulate the membrane potential and turgor pressure. The cytoplasmic K\(^+\) concentration is tightly controlled at values in the order of 100 mM. Vacuoles are the major sub cellular storage organelles for K\(^+\) in plant cells (Very and Sentenac 2002). In *Arabidopsis thaliana* at least 35 genes encode for K\(^+\) transport systems, out of these 15 encode for vacuolar and plasma membrane channels (Maser et al. 2001). The potassium channels are structurally classified mainly into three different families. They are shaker like potassium channels, K\(^+\) inward rectifying like channels and two pore potassium channels. Although shaker like potassium channels are not localized at the vacuolar membrane, the reasons for including them in this part are the following (1) to give a general view on potassium channels; (2) the slow vacuolar channel (see SV channel section below, which is the channel of interest in this thesis) shares some structural similarities with shaker like channel.

**Shaker like potassium channels**

The first evidence of the existence of a shaker like plant potassium channel was reported in 1992 (Sentenac et al. 1992). The name shaker like is given to this family of channels as they resemble the shaker potassium channel cloned from *Drosophila melanogaster* (Papazian et al. 1987). The shaker channels are tetramers i.e. four subunits tetramerize to form functional ion channel with a central pore forming unit. They have six transmembrane domains and one pore loop. The first four transmembrane domains form the voltage sensor unit, and the last two domains
Fig. 1.4. Topology and functional types of potassium channels in plants: the transmembrane domains and pore regions of three families (shaker like, TPK, Kir like) are shown on the upper panel. Abbreviations ext: external, mb: membrane and cyt: cytoplasmic side; P, CNBD, Anky, K\textsubscript{HA}, EF, respectively, pore domain, putative cyclic nucleotide-binding domain, ankyrin domain (i.e. domain rich in hydrophobic and acidic residues and Ef hand domain; +++; positively charged amino acids. The lower panel shows the current-voltage (I-V) relationships illustrating the functional types forming inward rectifying, outward-rectifying and weakly rectifying conductances of shaker type channels. TPK channels, missing the first four segments, are not regulated by voltages and show leak like conductance. Similar behavior is expected from Kir like channels. However, none of them has been functionally identified, so far. From Lebaudy, Very et al. (2007).

Form the pore region. Shaker type channels show inward, outward and weakly inward rectifying properties (see Fig. 1.4). There are excellent reviews available in the literature on shaker channels (Gambale and Uozumi 2006; Lebaudy et al. 2007; Borjesson and Elinder 2008).

**Two-Pore K\textsuperscript{+} channels**

Five different genes encoding TPK-type channels are present in *Arabidopsis thaliana* plants. AtTPK4 is localized at the plasma membrane (Becker et al. 2004) while the other four (AtTPK1, AtTPK2, AtTPK3 and AtTPK5) are localized at the vacuolar membrane (Voelker et al. 2006). TPK channels have four transmembrane domains and two pore loops and do not contain the voltage sensor domain (see Fig. 1.4). Consequently, membrane voltages do not regulate TPK channels and therefore they show a leak-like conductance. Some members of this family have EF hand domains (helix-loop-helix
motif binding Ca\textsuperscript{2+} ions) at the cytoplasmic side. It has been demonstrated that 14-3-3 proteins enhance the calcium-dependent TPK1 channel activity (Latz et al. 2007).

**K\textsuperscript{+} Inward Rectifying like channels**

The name Kir-like was given to this family as they resemble animal K\textsuperscript{+} Inward Rectifying channels. The first Kir-like channel in plants was identified in *Arabidopsis* by sequence related genome search (Czempinski et al. 1999). The Kir-like channel has two transmembrane domains and one pore loop. This channel has an EF hand domain at the C terminal end and does not contain a voltage sensor domain.

**Fast Vacuolar channel**

FV channel was first functionally identified in beet root vacuoles (Hedrich and Neher 1987), later in broad bean guard cell vacuoles (Allen and Sanders 1996) and in *Vicia faba* guard cell vacuoles (Allen et al. 1998). They are active at sub millimolar calcium concentrations at the cytoplasm. FV channel mainly conducts K\textsuperscript{+} due its availability but it is poorly selective between monovalent cations (Bruggemann et al. 1999). It has been reported that FV channel can be modulated by cytosolic and vacuolar potassium (Pottosin and Martinez-Estevez 2003). The molecular identity of FV channel is not yet revealed.
The **Slow Vacuolar channel**

The SV channel is one of the most extensively studied vacuolar channels. The SV channel was shown to be Ca\(^{2+}\) activated in vacuoles from beet storage tissues (Hedrich and Neher 1987) and has subsequently been identified in vacuoles from many plant cell tissues and types (Pantoja et al. 1989; Maathuis and Prins 1990; Colombo et al. 1994; Allen and Sanders 1997; Carpaneto et al. 1997; Paganetto et al. 2001; Scholz-Starke et al. 2004; Scholz-Starke et al. 2005a; Dziubinska et al. 2008). Nowadays it is generally accepted that the SV channel is ubiquitous in higher plants.

Features of the SV channel are the slow time of activation, and the outward rectification at elevated cytoplasmic Ca\(^{2+}\) concentration. For reviews see Scholz-Starke et al. (2005b) and Pottosin and Schonknecht (2007). The SV channel is a non-selective cation channel permeable to both monovalent and divalent cations (Pantoja et al. 1992; Ward and Schroeder 1994; Allen and Sanders 1996; Gambale et al. 1996; Ivashikina and Hedrich 2005).

It displays a main conductance, which depends on potassium concentration. The whole vacuolar currents of the SV channel investigated by patch-clamp method are in the order of 10 to 100 pA pF\(^{-1}\) in red beet storage root and 100-500 pA pF\(^{-1}\) in guard cells at +100 mV potentials (Allen and Sanders 1997). More precisely, SV single-channel conductance determined in sugar beet vacuoles are 40–300 pS at K\(^{+}\) concentrations ranging from 50 to 600 mM (Gambale et al. 1996) and 95 pS at 150 mM K\(^{+}\) (Scholz-Starke et al. 2005b). It has been suggested that the SV channel is permeable to both K\(^{+}\) and Ca\(^{2+}\) ions and that it may act as a Calcium Induced Calcium Release (CICR) channel (Ward and Schroeder 1994). Interestingly cytosolic magnesium also plays a role in the modulation of this channel (Pei et al. 1999; Carpaneto et al. 2001).

With densities of ~1 channel per µm\(^{2}\), or higher, the SV channel is the most abundant vacuolar channel in plants (Schulz-Lessdorf and Hedrich 1995; Pottosin et al. 1997). On the basis of conductance of single channel transitions the SV-channel pore-size is estimated to be around 7 Å (Amodeo et al. 1994; Paganetto et al. 2001).

In physiological conditions the tonoplast membrane potentials are slightly negative; consequently the physiological role of the SV channel is not clear so far, as the channel is mainly open at unphysiological positive tonoplast potentials. Therefore many studies are aimed at verifying whether any cellular parameter is able to modify or shift the voltage...
activation threshold of this channel towards more physiological negative potentials. Several possible agents such as cytosolic Ca\(^{2+}\) (Hedrich and Neher 1987; Reifarth et al. 1994), cytosolic pH, oxidising and reducing agents (Schulz-Lessdorf and Hedrich 1995; Carpaneto et al. 1999), cytosolic magnesium (Pei et al. 1999; Carpaneto et al. 2001), vacuolar Ca\(^{2+}\) (Pottosin et al. 2004), potassium gradient across the tonoplast (Ivashikina and Hedrich 2005), and cytosolic metals (Hedrich and Kurkdjian 1988; Corem et al. 2009) were shown to modulate the SV channel properties. It was reported that also pharmacological compounds, like TEA (Weiser and Bentrup 1993; Dobrovinskaya et al. 1999) and neomycin (Scholz-Starke et al. 2006) can modulate the channel activity.

**Structure of the SV channel**

In 2005 it was demonstrated that the gene encoding for the SV channels in *Arabidopsis thaliana* is AtTPC1 (At4g03560) (Peiter et al. 2005), whose molecular identity and topology were already reported in 2001 (Furuichi et al.). AtTPC1 is a unique gene in *Arabidopsis thaliana* plants where it is highly expressed in all tissues (Furuichi et al. 2001).

![Putative topology of AtTPC1 showing transmembrane segments (S1 to S12), pore domains (P1 and P2) and EF hands.](image)

AtTPC1 gene encodes a protein of 733 amino acids, which is targeted to the vacuolar membrane. It has two conserved homologous domains both of which contain six transmembrane segments (S1 to S6), a pore loop (P) between the S5 and S6 segments and a EF hand domain at the cytosolic side (see **Fig. 1.5**). The protein reminds two Shaker units which are fused together.
**Fig. 1.6.** Protein sequence of Arabidopsis thaliana TPC1 (AtTPC1) presenting putative transmembrane domains S1 to S6 (highlighted in yellow), putative pore domains P1 and P2 (in light green), and EF hands (in blue).

*Arabidopsis thaliana* TPC1 (AtTPC1) protein sequence is shown in **Fig.1.6.**
Some possible hypothesis on the physiological relevance of the SV channel

Even though the SV is the most extensively studied channel in plant vacuoles, its physiological role is not fully understood. It’s well known that the SV currents recorded in a typical patch-clamp experiment require unphysiologically high cytosolic and low vacuolar calcium concentrations for full activation. It was also reported that the channel is involved in calcium signalling thus suggesting that the SV channel is directly involved in Calcium Induced Calcium Release (CICR) in Arabidopsis thaliana where it might be involved in germination and stomatal movements (Peiter et al. 2005). However it has also been observed that SV CICR is incompatible with the drastic decrease of the open probability of the channel in conditions that favour the calcium release from the vacuole to the cytosol (Pottosin et al. 1997). Interestingly, the knock out plants of tpc1-2 do not show morphological differences compared to wild type plants. It seems that these plants do not suffer the loss of AtTPC1 gene.

Interestingly a point mutation in the vacuolar side of the channel determines different properties of the SV channel and even a diverse plant morphology, compared to wild type plants (Bonaventure et al. 2007). Indeed the Arabidopsis mutant fatty acid oxygenation up regulated 2 (fou2) is shown to up regulate oxylipin in the Jasmonate pathway. For a brief introduction to this pathway see section Ch. 1.4. In the fou2 mutant plant metabolites of 13-LOXs, and specifically oxylipin biogenesis strongly increased in response to wounding. In the fou2 mutant plants not only SV channel is active at more negative voltages but also the plants are more resistant to fungal pathogen Botrytis cinerea (Bonaventure et al. 2007). Moreover inhibition by vacuolar calcium is less pronounced in comparison with wild type plants as reported by Beyhl et al. (2009).

Recently it has been reported that Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) mobilizes calcium from acidic organelles through a two-pore channels from mammalian systems (Calcraft et al. 2009; Zong et al. 2009). NAADP is now an emerging molecule to study the calcium release from acidic stores through two-pore channels (for reviews see (Galione and Churchill 2002; Brailoiu et al. 2009; Guse 2009). The modulation of plant two-pore channel by this molecule still deserve further investigation.
Chapter 1.3. **Flavonoid biosynthesis, localization and transport**

Flavonoids are pigments or secondary metabolites present in all plants. They are one of the largest group of secondary metabolites, with more than 8000 different compounds, reported till now (Harborne and Williams 2000; Ververidis et al. 2007). The common structure of all the flavonoids is a C15 phenylchromane core composed of C6-C3-C6 backbone. The chromane (benzopyran) motif is made up of 2 condensed rings, an aromatic (benzo) A-ring (C6) and a heterocyclic (pyran) C-ring (C3) associated with another aromatic B-ring (C6). In the majority of flavonoids (flavanols, anthocyanidins, flavanones, flavones, and flavonols) the B-ring is attached at position 2, whereas in isoflavonoids the B-ring is instead attached at the position 3.

![Fig. 1.7. Basic structure of flavonoids. C15 phenylchromane core composed of C6-C3-C6 backbone. The chromane motif is made up of 2 condensed rings, an aromatic A-ring (C6) and a heterocyclic C-ring (C3) associated with another aromatic B-ring (C6). In the majority of flavonoids the B-ring is attached at position 2 (A), whereas in isoflavonoids the B-ring is attached at position 3 (B).](image)

Each flavonoid subclass comprises numerous members, differing in the degree of hydroxylation or methoxylation of A and B rings. Modifications at single or multiple positions of flavonoid molecules via hydroxylation, methylation, acylation, or glycosylation reactions provide unique features and characteristics to most of the currently known flavonoids (Fig. 1.8). For review on flavonoid biosynthetic pathway see Winkel-Shirley (2001), Lepiniec et al. (2006) and Winkel-Shirley (2006).
Fig. 1.8. Flavonoid biosynthetic pathway. ACCase, acetyl CoA carboxylase; ANS, anthocyanidin synthase; AS, aureusidin synthase; DFR, dihydroflavonol 4-reductase; DMID, 7,2′-dihydroxy, 4′-methoxyisoflavanol dehydratase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; F3’5’H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; FSI/FS2, flavone synthase; I2′H, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; IFS, isoflavone synthase; IOMT, isoflavone O-methyltransferase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; OMT, O-methyltransferase; RT, rhamnosyl transferase; UFGT, UDP flavonoid glucosyl transferase; VR, vestitone reductase. From Lepiniec et al. (2006).
Flavonoids are secondary metabolites unique to plants, where they play very important roles in many processes such as defense against insects (Simmonds 2003; Lev-Yadun et al. 2004; Treutter 2006), UV protection (Li et al. 1993; Kootstra 1994), antioxidant activity (Rice-Evans et al. 1997; Hernández et al. 2009), temperature stress (Coberly and Rausher 2003; Kirakosyan et al. 2003), heavy metal tolerance (Cocker et al. 1998; Kidd et al. 2001), chemical signals for pollination (Grotewold 2006), pollen growth (Taylor and Grotewold 2005; Yang et al. 2008), seed dormancy and seed protection (Debeaujon et al. 2000), microbial interactions (Cohen et al. 2001; Shaw et al. 2006) and regulation of auxins for root development (Imin et al. 2007; Santelia et al. 2008).

It is generally believed that flavonoids are synthesized through the phenylpropanoid pathway, by cytosolic multienzyme complexes at the cytoplasmic surface of the Endoplasmic Reticulum (ER) (Stafford 1974; Winkel-Shirley 1999). Some enzymes, participating in flavonoid metabolism, were also reported to co-localize at the ER, tonoplast, nucleus, secondary cell wall and plastid (Saslowsky and Winkel-Shirley 2001; Tian et al. 2008). The flavanone Naringenin plays a central role in the biosynthetic pathway (see Fig. 1.8) and is present in all the plants; in plants such as Citrus is one of the major flavonoids (Rouseff et al. 1987). The glycosylase transferase gene, which modifies Naringenin into Naringin, was also reported to be present in Citrus fruits (Frydman et al. 2004). It has been reported that Naringenin is involved in the fungal resistance (Padmavati et al. 1997), in lateral root growth (Webster et al. 1998) and in lignin biosynthesis (Deng et al. 2004) in plants. Naringin is evolved in microbial interactions (Cohen and Yamasaki 2000). Flavonoid deficient mutants are useful tools to study the importance of flavonoids in plants. They are grouped into transparent testa (tt) phenotype mutants, where the seed coat is affected at various steps (see Fig. 1.9) by a deficiency in the flavonoid biosynthetic pathway (Shirley et al. 1995; Buer and Muday 2004; Buer and Djordjevic 2009).
Fig. 1.9. Schematic representation of flavonoid biosynthetic pathway. Enzymatic steps affected in specific transparent testa mutants are indicated with putative regulatory loci indicated in parentheses. From Shirley et al. (1995).
Flavonoids are transported to different locations from the sites of their synthesis. Most of the flavonoids are accumulated in the vacuole; others may be secreted and incorporated into the cell wall. Even though the major steps in flavonoid biosynthetic pathway are known, there are still unanswered questions concerning the mechanism(s) by which the flavonoid end-products are properly sorted and transported into the diverse intracellular compartments.

Fig. 1.10. Hypothetical scheme of flavonoid transport pathways in grapevine. Flavonoids could be conjugated with glutathione (GSH) through a reaction catalysed by glutathione S transferases (GSTs). The main transporters localized in grapevine vacuole and plasma membrane are the ATP-binding cassette (ABC) proteins and the bilitranslocase-homologue (BTL-homologue). The multidrug and toxic compound extrusion (MATE) protein, shown to be involved in flavonoid transport in other plant species, has also been added. Transport mediated by vesicle trafficking is indicated by circles (AVIs, anthocyanic vacuolar inclusions; ACPs, anthocyanoplasts). Question marks indicate the lack of information or still hypothetical components and steps in the process. From (Braidot et al. 2008).

It has been suggested that the flavonoids are transported to the vacuolar membrane through different pathways (Kitamura 2006; Braidot et al. 2008; Passamonti et al. 2009) (see Fig. 1.10). In one of the mechanisms it is assumed that flavonoid-specific Glutathione S Transferases (GSTs) carry flavonoids to the vacuole via non-covalent
activity (Grotewold 2001). Alternatively, the second mechanism requires a complex vesicle-trafficking network, possibly involving the Golgi apparatus (Grotewold 2004; Grotewold 2006; Poustka et al. 2007). Specific vacuolar transporters were also reported from several species such as maize (*Zea mays*), petunia (*Petunia hybrida*), *Arabidopsis thaliana* and grapevine (*Vitis vinifera*) (Kitamura 2006). These transporters can be broadly divided into two types: those, which are activated by the hydrolysis of ATP, and those dependent on a secondary activation, modulated by the trans-membrane proton gradient across the vacuolar membrane. The ATP-binding cassette (ABC) proteins were shown to be involved in the transport of flavonoids into the vacuolar lumen (Yazaki 2005; Klein et al. 2006; Yazaki 2006; Rea 2007). In contrast proton/flavonoid antiporters use trans-membrane proton gradients to transport flavonoids into the vacuole. Multidrug And Toxic Efflux (MATE) proteins mediate this mechanism as reported in *Arabidopsis thaliana* (Debeaujon et al. 2001; Marinova et al. 2007b) and in barley (*Hordeum vulgare*) (Marinova et al. 2007a). It has been suggested that Glutathione-linked flavonoids would be primarily recognized by ABC proteins, in particular Multidrug Resistance associated Proteins (MRP) (Yazaki 2005) and glycosylated flavonoids would be preferentially transported by secondary active MATE-like antiporters.

Ch.1.4. Polyunsaturated fatty acids

Membrane lipids are classified into phospholipids, cholesterol and glycolipids and their ratio may differ from membrane to membrane. All of them possess polar regions typically facing towards the aqueous compartments and non-polar regions which constitute the interior of the lipid bilayer. Membrane phospholipids are built upon the three carbon backbone of glycerol and have a polar phosphate group or phosphate plus a polar, hydrophilic group in the *sn*-3 position and two fatty acid tails. The fatty acid tail esterified to the first carbon of the glycerol backbone (*sn*-1 position) is generally a saturated fatty acid, lacking carbon–carbon double bonds. The tail esterified to the second carbon (*sn*-2 position) is generally an unsaturated fatty acid with one (monounsaturated) or more (polyunsaturated) carbon–carbon double bonds.
The mammalian membrane phospholipids mainly contain long chain ω-3 (eicosapentaenoic acid, EPA, C20:5) and ω-6 (Arachidonic Acid, AA, C22:6) fatty acids. As they serve as signalling molecules many studies are aimed at verifying modulation of mammalian ion channels by polyunsaturated fatty acids (PUFAs). We know for almost 20 years that lipids and lipid bioproducts are able to fine-tune voltage-gated ion channels in animal cells. For review on this topic see Boland and Drzewiecki (2008). Little or no Arachidonic Acid is present in plants however plants use other polyunsaturated fatty acids such as linoleic acid and Docosahexaenoic acid. For example the plant phospholipids of tonoplast mainly contain ω-6 (linoleic acid, C18:2). In fact a study demonstrated that more than 50% of the total fatty acid composition in the vacuolar membrane of the plants parsnip, parsley and carrot is made of linoleic acid (Makarenko et al. 2007). The interaction of PUFAs with plant ion channels is less studied so far, but the downstream products of PUFAs, such as jasmonate members of oxylipins, (which lies in the wound response pathway) are in focus for the last few decades. The biosynthesis of most plant oxylipins are initiated by the action of lipoxygenases (LOX), which are capable of introducing molecular oxygen at either C19 or C13 position of the C18 fatty acids linoleic acid and α-linolenic acid (Feussner and Wasternack 2002). A mutation in the vacuolar side of the AtTPC1 gene (fou2 mutant) was shown to up regulate oxylipin and this mutant plants were more resistant to the fungal pathogen Botrytis cinerea (Bonaventure et al. 2007). For reviews on jasmonate pathway see Wasternack (2007) and Bottcher and Pollmann (2009).

Ch 1.5. Objectives

The Slow Vacuolar channel is one of the most extensively studied ion channels in plants. The SV channel was shown to be calcium activated in vacuoles from beet storage tissues (Hedrich and Neher 1987) and was subsequently identified in vacuoles from many tissues and plants (Allen and Sanders 1997; Carpaneto et al. 1997; Scholz-Starke et al. 2005a; Dziubinska et al. 2008), thus demonstrating that it is ubiquitous in higher plants. Features of the SV channel are the slow activation and the outward rectification at elevated unphysiological cytoplasmic calcium concentrations. Indeed it is well known
that the SV currents recorded in a typical patch-clamp experiment require unphysiologically high cytosolic and low vacuolar calcium concentrations for activation. Furthermore, it has been demonstrated that the SV channel, besides being permeable to potassium ions, is also permeable to calcium (Gradogna et al. 2009). It is well known that the Slow Vacuolar channel is modulated by several parameters and compounds, internal and external to the vacuole; beside calcium, Mg$^{2+}$, protons, reducing agents, metals (like Zn$^{2+}$ and Ni$^{2+}$) and the glycosidic antibiotic Neomycin are able to modify the properties of the channel. However, none of these parameters is able to activate the SV channel at physiological membrane potentials and calcium concentrations. For this reason we aim at investigating unexplored working conditions or compounds, which are able to further modulate this channel. Specifically we plan to find out endogenous plant molecules, such as flavonoids and PUFAs, which might be able to modify the voltage activation threshold of this channel towards more physiological conditions or that may provide information on the role and the nature of this enigmatic channel.
Materials and methods
Chapter 2.1. **Methods for localization of flavonoids in plant leaves**

**Plant material**

*Arabidopsis thaliana* seeds from Columbia (Col-0) and Landsberg erecta (Ler) ecotypes were sowed in soil pots. After 5 days of vernalization at 4°C they were transferred to the growth chamber for germination (light period was 12h dark and 12h light), 8-10 days after they were individually transferred in pots and grown under controlled conditions in the green house. The light period was 16h dark and 8h light, with a light intensity of 179 µmol m\(^{-2}\) s\(^{-1}\) and a temperature of 22°C. Plants were weekly watered with nutrient solution: 18% Nitrogen (12% Nitrate, 6% Ammonia), 6% Phosphorus Anhydride, 26% Potassium Oxide, 2% Magnesium Oxide and oligoelements <1%.

*Nicotiana tabacum* plants were grown on soil in 9 cm pots and kept under controlled conditions in growth chamber. The light period was 16h dark and 8h light, with a light intensity of 70 µmol m\(^{-2}\) s\(^{-1}\) and a temperature of 22°C.

**Construction of recombinant plasmids**

The AtGT (At1g06000) was amplified by PCR using the Phusion® DNA Polymerase (Finnzymes, Finland) and *Arabidopsis thaliana* genomic DNA was used as template, since no intrones are predicted in the gene sequence. The primers used for the PCR were the following: Nar-NcoI-For (5’-CATGccatggccATGACAACAACAACAACGAAGA-3’, right before ATG start codon with NcoI site) and Nar-NcoI-Rev (5’-CATGCcatggcTgcCAAACACATCTCTGCAACGAGCT -3’, after the stop codon with NcoI site). The stop codon TAA was mutated into GCA and, to maintain the frame, two extra CC where introduced between the AtGT and the Yellow Fluorescent Protein (YFP) coding sequences; in this way two alanines were introduced at the junction of the fusion protein. The PCR product was digested with NcoI enzyme and cloned in the vector pGreen 0029 in which previously multiple cloning sites was replaced with 2x35S promoter, YFP and nos terminator using KpnI and SacI sites (Zottini et al. 2008). The right orientation was confirmed by digestion and sequencing.
Western Blot
For protein extraction, tobacco leaf pieces, corresponding to agroinfiltrated areas, were harvested, frozen, powdered in liquid N\textsubscript{2} and homogenized in two volumes of protein extraction buffer [0.3 M sucrose, 0.1 M Tris pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 5 mM e-aminocaproic acid, 5 µg ml\textsuperscript{-1} leupeptin, 2 µg ml\textsuperscript{-1} aprotinin and 0.7 µg ml\textsuperscript{-1} pepstatin]. The homogenate was centrifuged at 1500 g for 15 min at 4°C to eliminate debris. Protein concentration was determined by the Bradford method, using the Bio-Rad protein assay (Bio-Rad, Segrate, Italy). Total extracted proteins (3 and 6 µg) were separated by 12% (w/v) SDS–PAGE, transferred to a nitrocellulose membrane (Sartorius) and hybridized with a polyclonal antibody raised against YFP (Invitrogen Molecular Probes). The blot was then hybridized with an alkaline phosphate conjugated secondary antibody and the detection was performed with the NBT/BCIP colorimetric assay.

Transient expression
A. Mesophyll protoplasts from *Arabidopsis thaliana*
The transient transformation was performed following the Sheen protocol (2002). Briefly leaves from soil grown 4/5 weeks old *Arabidopsis thaliana* plants were cut into narrow strips and put in freshly prepared enzymatic solution (1.25% cellulase R10, 0.03% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM CaCl\textsubscript{2} and 0.1% BSA, pH 5.7) under vacuum for 30 minutes. The digestion was then carried out for 3 hours at 25°C in the dark. The protoplasts were then filtered with 50 µm nylon mesh and centrifugated at 90 g. The pellet was washed twice with W5 solution (154 mM NaCl, 125 mM CaCl\textsubscript{2}, 5 mM KCl and 2 mM MES, pH 5.7) and the protoplasts were kept in ice for 30 min before the PEG transformation. PEG-mediated protoplast transformation was achieved on protoplasts aliquots of 10\textsuperscript{5} protoplasts/ml, with 4 µg of DNA. Transformed protoplasts were incubated overnight in W5 solution in the dark before confocal analysis.

B. Tobacco leaves
Competent cells of *Agrobacterium tumefaciens* GV3101 strain were prepared as previously described (Zottini et al., 2008) and the binary vectors were introduced by
freeze/thaw method. For the use of pGreen-derived vectors we cotransformed the *Agrobacterium tumefaciens* cells with the pSoup vector (Hellens et al. 2000a). The *Agrobacterium* is grown in YEP medium (Yeast Extract, 10 g/l; Bacto-Peptone 10 g/l; NaCl 5 g/l; pH 7.2) supplemented with specific antibiotics (rifampicin 25 mg/l, gentamycin 50 mg/l, kanamycin 50 mg/l and tetracycllin 5 mg/l) on a rotary shaker at 28°C.

**Agroinfiltration of tobacco leaves**

The tobacco agroinfiltration protocol was performed as described in Batoko et al. (2000). Two days prior to agroinfiltration, single colonies of *Agrobacterium tumefaciens* growing on agar plates were inoculated in 5 ml YEP liquid medium supplemented with specific antibiotics. The bacterial cultures were incubated at 28–30°C at 200 rpm on an orbital shaker. New bacteria cultures were started two days later by inoculating fresh medium with the old suspension cultures (1/200 ratio, v/v). The new cultures were grown under the same conditions for an additional day. After 24h, 2 ml of each bacteria culture were transferred to eppendorf tubes and pelleted by centrifugation at 1,500 g for 4 min. in a micro-centrifuge at room temperature. The pellets were washed twice with 2 ml of infiltration buffer [50 mM MES pH 5.6, 2 mM Na$_3$PO$_4$, 0.5% glucose (w/v) and 100 µM acetoxyringone (Aldrich, Italy)]. The bacteria suspensions were diluted with infiltration buffer to a final OD$_{600}$ of 0.2 and allowed to grow for 1-2h at 25°C in the dark, with continuous gentle shaking before using them for agroinfiltration experiments. The inocula were delivered to the lamina tissues of tobacco leaves by gentle pressure infiltration through the stomata of the lower epidermis by using a 1-ml syringe without a needle. Young leaves prior to full expansion were used. After infiltration the plants were transferred in a growth chamber under standard growth conditions.

**Fluorescence image acquisition**

YFP-dependent fluorescence was analyzed after 24 hours in case of *Arabidopsis* mesophyll protoplasts and 6 days after agroinfiltration of tobacco leaves. *Arabidopsis* protoplasts were mounted on the slides with cover slips for microscopic observations. Pieces of tobacco leafs were randomly cut from the infected areas and mounted on slides for microscopic observations. Confocal microscope analyses were performed using a
Nikon PCM2000 (Bio-Rad, Germany) laser scanning confocal imaging system. For YFP detection excitation was at 488 nm and emission between 530/560 nm. For the chlorophyll detection, excitation was at 488 nm and detection over 570 nm. Image analysis was done with the ImageJ bundle software (http://rsb.info.nih.gov/ij/).

Ch. 2.2. **Electrophysiology: the patch-clamp methodology**

Prior to 1976 typical electrophysiological experiments were performed on large cells, like the squid giant axon, which have a size in the order of about 1 mm x 2 cm (Hille 1978). Erwin Neher and Bert Sakmann introduced patch-clamp technique in 1976 (Neher and Sakmann 1976) in order to allow the measurement of ionic transport under controlled potential and ionic conditions in small (~10-100 μm) cells.

A glass pipette with a very small opening (<1 μm) is used to make tight contact with a tiny area, or patch, of the vacuolar membrane. After the application of a small negative pressure, the seal between the pipette and the membrane becomes so tight that very few ions can flow between the pipette and the membrane. In good conditions the electric resistance between the pipette and the membrane reaches values larger than one gigaohm, \(10^9 \Omega\) ("gigaseal" see Fig. 2.1). This arrangement is usually called the vacuole-attached patch-clamp recording configuration (Fig. 2.2 A). Consequently, the recorded current (flowing through the recording pipette) is exclusively the current passing through the membrane. Thus, all the ions that flow when a single ion channel opens must flow into the pipette. The resulting electrical current, though small, can be measured with an ultra sensitive electronic amplifier connected to the pipette.

Once a tight seal is attained, several experimental options are available, according to the type of currents that need to be studied (Fig. 2.2). If the membrane patch below the pipette is disrupted by applying a relatively strong suction pulse or a large voltage pulse, the interior of the pipette becomes continuous with the interior of the vacuole. This arrangement allows measurements of electrical potentials and currents from the entire vacuole and is therefore called the whole-vacuole recording configuration (Fig. 2.2 B). The whole-vacuole configuration also allows diffusion exchange between the pipette and the interior of the vacuole, producing a convenient way to perfuse substances into the interior of a “patched” vacuole.
**Fig. 2.1.** Schematic representation of a “gigaseal” during a patch-clamp experiment. A) When the pipette is still outside of the solution, the current consists of a fast capacitive transient. B) The pipette is dipped into the bath while applying slightly positive pressure, in order to keep the tip of the pipette clean while passing through the air/water interface. C) The pipette is still far from the vacuole, and the current, observed as a response to a small potential pulse, allows to evaluate the pipette resistance. D) The pipette is carefully approaching the vacuole, while still keeping a slightly positive pressure inside. A decline in the current indicates that the pipette is touching the vacuole. E) The pressure is released and a further decline of the current is observed. F) A weak negative pressure is applied until the seal is formed. The formation of a good seal is pointed out by an almost complete decline of the current. The reduction of the leak current reduces also the registration’s background-noise, allowing single channel registration. A contact achieved in this way presents high mechanical stability, that allows to perform registrations in four different configurations.
Fig. 2.2. Schematic representation of four configurations that can be achieved during a patch-clamp experiment. A) “Vacuole-attached” configuration; B) “Whole-vacuole” configuration, depart from a vacuole-attached configuration; C) “Inside-out” patch configuration, depart from a vacuole-attached configuration; D) “Outside-out” patch configuration, depart from a whole-vacuole configuration.

If the pipette is retracted while it is in the whole-vacuole configuration, the membrane patch obtained has its cytosolic surface exposed to the bath. This arrangement, called the outside-out recording configuration (Fig. 2.2 D), is optimal for studying how the single channel activity of one or a few channels is influenced by cytosolic chemical signals. Once a tight seal has formed between the membrane and the glass pipette, small pieces of membrane can be pulled away from the cell without disrupting the seal; this yields a preparation that is free of the complications imposed by the rest of the vacuole. By retracting a pipette that is in the vacuole-attached configuration one generates a small patch of membrane with its vacuolar surface exposed to the bath solution. This arrangement, called the inside-out patch recording configuration (Fig. 2.2 C), allows the measurement of single-channel currents with the added benefit of making it possible to change the medium to which the surface internal to the vacuole is exposed.
The patch-clamp setup

A patch-clamp setup (Fig. 2.3) consists of a microscope placed on an antivibrational table within a Faraday cage, that reduces to a minimum the electric disturbances from the surrounding electronic instruments. The system contains also a patch-clamp amplifier for voltage-clamping the cell, a micromanipulator holding the amplifier probe and the attached patch-pipette and a personal computer to generate the stimulation and record the current. An inverted microscope (Zeiss Axiovert 10) with a magnification of up to 320X (32X10), allows the visualization of the cell and to monitor the touch-down of the pipette on the cell.

In order to obtain good seals, it is essential to precisely control the movement of the pipette in the sub micrometer range. To this purpose two micromanipulators are used: 1) a mechanically driven system (Microcontrole, France), allowing movements in the order of mm, to be used when the pipette is still far from the vacuole and 2) a hydraulically driven system (Narishige, Japan), allowing fine movements in the order of fractions of micrometer. The stimulation and acquisition system is based on a personal computer (Macintosh Quadra 700), which uses a specially designed software (Pulse+PulseFit, Heka Electronics, Germany) to send to the amplifier the stimulation protocols and register the evoked currents. An A/D/A converter (Instrutech ETC 16) provides the in and out data conversion.

The membrane potential and the survey of the current signal are controlled by a patch-clamp amplifier (List EPC-7), which has two parts: the headstage and the central body. The headstage is equipped with an electrode-holder; this is the physical support for the glass pipette, and has a double role: firstly, to establish the electrical contact between the pipette solution and the current/voltage converter of the headstage through an Ag||Ag/Cl electrode and secondly, to allow the regulation of the pressure applied to the cell through the glass pipette.
Fig. 2.3. Patch-clamp setup at the IBF-CNR laboratories in Genoa. At the right hand side the computer monitor can be seen. In the center, the electronic equipment, from bottom: A/D/A converter, filter, central body of the amplifier. At the left hand side: Faraday cage, where the microscope, the amplifier-headstage and the micromanipulators are positioned. The microscope is equipped with a video camera transferring images to the monitor on the top of the Faraday cage.

In the headstage the current signal is converted to a voltage signal and transferred to the amplifier. The amplifier allows also to change the gain, and to compensate the effects of the pipette capacity, the membrane capacity and the access resistance. Currents from several tens of nA to fractions of pA can be recorded by the amplifier. The voltage signals are filtered by a four-poles Bessel filter (KEMO) and transferred to the computer for digital display and data storage on the hard disk.
Perfusion systems

During the patch-clamp experiments the ionic solution of the bath needs to be changed from the standard (control) solution to different ionic solutions.

In this work, two types of perfusion systems were used: a “gravity-driven” and a “fast” perfusion system (Carpaneto et al. 1999; Carpaneto et al. 2001), that differ mainly in the speed, of changing the external solution.

![Image of perfusion system with annotations](image.png)

Fig. 2.4. The registration-dish placed on the microscope, with arrows indicating (1) the electrode-holder with the glass micro-pipette, (2) the small tubes of the perfusion system, (3) the suction-tube of the peristaltic pump and (4) the ground-electrode.

A gravity-driven perfusion system allows to change the external solution in a few minutes by means of 2-6 syringes of 20 ml (depending on the experimental demand). The syringes deliver the solution by means of a valve and polyethylene tubes (Fig. 2.4). The bath solution is constantly removed from the registration petri dish by means of a peristaltic pump.

A fast-perfusion system is used to change the external solution in time intervals in the order of few ms. The system comprises a part that regulates the velocity of the flux, which includes a support system with an independent height regulation (with respect to the registration petri-dish) for 3 capillaries of 200 μl. The capillaries are treated with
**Fig. 2.5.** Schematic representation of the “fast perfusion system” (above); a picture through a microscope showing 3 pipettes containing different solutions, positioned close to the patched vacuole (below).

the silanizing agent sigmacote that decreases the surface tension of the liquid bathing the interior of the pipette. The capillaries are connected to a valve on one side, and to a flexible tygon tube on the other side. The second part is formed of 3-glass micropipette with a tip of 20 to 30 μm diameter. On one side they are connected to the rest of the system with a tygon tube, while on the other side they are soaked in the bath solution (Fig. 2.5). The 3 micropipettes are fixed to a metal support that allows an independent regulation of their position in three directions, while they are lined in the same focal plane and the distance between them is about 10 μm. The perfusion pipette can be positioned in front of the cell by means of hydraulic micromanipulator (Narishige, Japan).

Instead of plastic tubings, glass tubes connected to oil-filled syringes controlled by a stepping motor was used for the perfusion of polyunsaturated fatty acids (PUFAs) to avoid the binding of lipids to the perfusion system. This system allowed a slow release of the PUFA solution with a flow rate of about 10 μl/min.

**Micropipettes and electrodes**

The patch pipettes were pulled by a pipette puller (Micropipette puller mod L/M 3P-A List Medical) from borosilicate glass capillaries (Clark Electromedical Instruments) which have an external diameter of 1.7 mm and an internal of 1.2 mm. The capillaries were cleaned by flame and coated internally with sigmacote.
Data acquisition

Patch-clamp experiments were typically made in excised-patch configuration. Patches in the excised configuration are much more stable than those in the whole-vacuolar configuration and allow to maintain the patch for a long time under fast or slow perfusion conditions. Pipette resistance was 1.8-2.4 mOhm. Seals with more than 2 GOhm resistances were obtained prior to the establishment of the excised-patch configuration. Patch-clamp recordings were made by an EPC7 (List) amplifier or in alternative by an Axon 200A amplifier (Axon Instruments). Data were acquired with software Pulse and Pulsefit (HEKA) and analysed with Igor Pro (Wavemetrics). Currents are typically evoked in response to series of +10 mV voltage steps ranging from -80 to +120 mV; tail voltages are the voltages following the main step pulse (Fig. 2.6). The signs of current and voltage follow the convention proposed by Bertl et al. (1992).

![Diagram of voltage pulse](image)

Fig. 2.6. Typical stimulation protocol used to record the ionic current at different step potentials. The “tail” currents are elicited by “tail” voltages applied after the main pulse. In our working conditions the tail voltage is typically equal to the holding potential (-50 mV; see Fig. 3.4 for SV currents elicited by this protocol).

Data analysis

The I-V characteristics of the SV channel is constructed by plotting the average value of the current recorded during the last 50 ms at each applied voltages.
The dose-response analysis of Naringenin block was performed by calculating the residual SV currents \( \frac{I_{\text{Nar}}}{I_{\text{control}}} \) at +80 mV. \( \frac{I_{\text{Nar}}}{I_{\text{control}}} \) was plotted as a function of Naringenin concentration and data were fitted with the Hill equation

\[
\frac{I_{\text{Nar}}}{I_{\text{control}}} = \frac{1}{1 + ([\text{Nar}] / K_h)^n} \tag{2.1}
\]

where \([\text{Nar}]\) is the Naringenin concentration, \(K_h\) and \(n\) are the Hill inhibition constant and Hill coefficient, respectively.

In the dose-response analysis where \(G_{\text{norm}}\) was plotted as a function of Nar or AA concentrations \(G_{\text{norm}}\) was obtained from the Boltzmann analysis (see below).

**Boltzmann analysis:**

1. We define \(I_t\) as the current at the beginning of the tail protocol (see protocol in Fig.2.6 and Fig 3.4). The equation for \(I_t\) is the following:

\[
I_t(V_p) = N \cdot g(V_t) \cdot (V_t - V_{\text{rev}}) \cdot P(V_p) \tag{2.2}
\]

where \(V_p\)=voltage of main pulse, \(N=\)number of channels, \(g=\)single channel conductance, \(V_t=\)tail potential, \(V_{\text{rev}}=\)reversal potential, \(P(V_p)=\)open probability at the voltage of main pulse.

2. Evaluation of \(I_t\): the tail current was fitted with a single exponential function. The fit was performed 1-3 ms after the onset of the tail voltage in order to remove capacitance artefacts.

3. We fitted \(I_t(V_p)\) with the following Boltzmann function:

\[
I_t = I_{\text{off}} + I_{\text{max}} \cdot (1 + \exp \left( \frac{- (V_p - V_{1/2})}{s} \right)) \tag{2.3}
\]

where \(I_{\text{off}}, I_{\text{max}}, V_{1/2}\) and \(s\) are the free parameters of the fit. \(I_{\text{off}}\) represents the current offset, \(I_{\text{max}}\) the maximum of the current, \(V_p\) the applied voltage, \(V_{1/2}\) half activation voltage, and the \(s\) is \(RT/Fz\) where \(z\) is the apparent gating charge.

4. Normalized conductance was calculated by
\[ G_{\text{norm}} = \frac{(I_t - I_{\text{off}})}{I_{\text{max}}(\text{control})} \]  
\[ \text{eq. 2.4} \]

where \( I_{\text{max}}(\text{control}) \) is the maximum value of the Boltzmann fit in control conditions.

5. Averages of \( G_{\text{norm}} \) for at least three different vacuoles in control and at varying concentrations of Naringenin and Arachidonic Acid were calculated. Half activation time is the time at which the current assumes the half of its maximum value. The maximum value is evaluated from the difference between steady state current, and the initial current, where the channels are still closed.

**Detection of fura2 fluorescence and data analysis**

The recording chamber was mounted on the stage of an inverted microscope (IM35, Zeiss, [http://www.zeiss.com/](http://www.zeiss.com/)) with a 100× Nikon Fluor objective (Nikon, [http://www.nikon.com](http://www.nikon.com)). For fura-2 excitation, the tip of the recording pipette was illuminated using a xenon lamp via a light fiber. The light beam passed through a rotating wheel with six interference filters, four centered at 340 nm and two centered at 380 nm. A computer-driven spectrophotometer system (Cairn Research, [http://www.cairn-research.co.uk](http://www.cairn-research.co.uk)) controlled both rotor speed and acquisition of emission light through a photomultiplier (Fig. 2.7). The mean emission relative to each set of filters was calculated. The speed was set at 32 rotations per second (rps), and the final recording frequency was one point every second. Part of the experiments was repeated using a new set of interference filters, one centered at 340 nm and one centered at 380 nm.
**Fig. 2.7.** Schematic representation of the experimental set up for the fluorescence measurements.

In this case, the speed was set at 20 rps, the final recording frequency was one point every 50 msec, and the traces reported in the figures were filtered off-line at 1 Hz. There was no difference between the results of the two sets of experiments. The voltage applied through the patch–clamp system was recorded simultaneously with the fluorescence signals by the A/D board of the computer controlling the spectrophotometer. **Fig. 2.8** illustrates the raw data of the fluorescence signals after background correction.
All segments of the 380 nm component in the absence of transients throughout the experiment (marked in black) were fitted with a polynomial function (black line). Amplitudes of both fluorescence components were then normalised to the resulting polynomial.
Step by step procedure
Pulling of the patch pipette and bending of the very tip by 45° using a flame. Bending serves to compensate for the 45° of the holder in order bring the pipette tip in a quasi-vertical position and to obtain a symmetrical pipette orientation in the focal plane.
Back-filling of the pipette with pipette solution containing the fluorophore fura-2 (at a concentration of 100 µM) in the dark. Generally, all operations handling fura-2 are done in red ambient light to limit photobleaching.
High-resistance seal is obtained on isolated vacuoles. After reaching the whole-vacuole configuration using combined voltage pulse (700 mV, 700 µs) and slight suction, patch excision leads to the cytosolic side-out configuration.
The tip of the patch pipette is set into the focal plane of the microscope in bright field (equipped with a red-light filter). With excitation light and photomultiplier on, the focal plane is slightly adjusted (usually shifting upwards), in order to gain comparable fluorescence signals between different experiments.
Simultaneous recordings of ionic currents and fluorescence signals upon voltage stimulation are performed. Bath solutions are changed by means of a gravity-driven perfusion system. The chamber volume of 0.1 ml is normally exchanged at least 20 times, at a speed of about 1 ml*min⁻¹ (slow perfusion procedure).
At the end of the experiment, background fluorescence is measured after setting the focal plane far from the pipette; voltage offset is measured after removal of the membrane patch by bringing the pipette tip in contact with the bottom of the recording chamber.

Protoplast and vacuole isolation
From Arabidopsis:
For isolation of mesophyll protoplasts, rosette leaves of 3–5 week old plants were used. After careful removal of the lower epidermis, leaves were incubated for 30 min at 30°C in enzyme solution containing 0.3% Cellulase, 0.03% Pectolyase Y-23 (Seishin), 1 mM CaCl₂, 1 mM MgCl₂, 10 mM MES pH 5.5 with KOH, n=600 mOsm with sorbitol. Protoplasts were washed twice and resuspended in maintaing solution containing 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7 with KOH, n=600 mOsm with sorbitol, by centrifugation at 90 g for 1 min. Protoplasts were kept in the maintaing solution at room temperature until used for experiments.
Vacuole release was observed under the microscope after adding EGTA to aliquots of protoplast solution. The concentration of EGTA needs to be optimised to release vacuole without compromising their stability. After settling of the vacuoles, solution was carefully replaced by bath solution. Typically large and clear vacuoles are patched.

**From carrots:**
Fresh carrots were supplied by a local company and were used on the same day of the experiments. Carrot taproot parenchymal tissue vacuoles were readily extruded into the recording chamber by gently slicing the root cortex tissue into the standard bath solution.

**Experimental solutions adopted in this work**

**Excised cytosolic side-out experiments:**

**Standard control solution 1:** For the recordings of macroscopic currents standard, ionic solutions were as follows: 200 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), and 10 mM HEPES/Tris, pH 7.5 in the bath. DTT was added to the bath solution in order to prevent channel rundown (Carpaneto et al. 1999; Scholz-Starke et al. 2004). The osmolarity in both solutions was adjusted to 600 mOsm by the addition of D-sorbitol.

**Standard control solution 2:** 200 mM KCl, 2 mM MgCl₂, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT) and 10 mM HEPES/Tris, pH 7.5 in the bath. The osmolarity in both solutions was adjusted to 600 mOsm by the addition of D-sorbitol.

**Standard control solution 3:** 200 mM KCl, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 1 mM CaCl₂, 1 mM dithiothreitol (DTT) and 10 mM HEPES/Tris, pH 7.5 in the bath. The osmolarity in both solutions was adjusted to 600 mOsm by the addition of D-sorbitol.

**Standard control solution 4:** For recordings of single SV channel conductance, standard solutions were as follows: 100 mM KCl, 20 mM HEPES/5 mM KOH, pH 7.0 and 100 µM fura-2 in the pipette; 100 mM KCl, 2 mM CaCl₂, 2 mM dithiothreitol (DTT) and 20
mM HEPES/5 mM KOH, pH 7.0 in the bath. The osmolarity in both solutions was adjusted to 420 mOsm by the addition of D-sorbitol. Free calcium in this solution (considering a Ca\(^{2+}\) contamination of 2.5 µM measured by atomic absorption spectroscopy) was estimated to be 5 nM (http://maxchelator.stanford.edu).

**Excised vacuolar side-out experiments:**

**Standard control solution 5:** For recordings of macroscopic currents in vacuolar side-out patch configuration, standard solutions were 100 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 1 mM dithiothreitol (DTT) and 10 mM HEPES/Tris, pH 7.5 in the pipette; 200 mM KCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM MES/Tris, pH 5.5 in the bath. The osmolarity in both solutions was adjusted to 600 mOsm by the addition of D-sorbitol.

**Standard control solution 6:** 100 mM KCl, 1 mM CaCl\(_2\), 1 mM dithiothreitol (DTT) and 20 mM HEPES/1 M KOH, pH 7.0 in the pipette; 100 mM KCl, 1 mM CaCl\(_2\), 20 mM HEPES/1 M KOH, pH 7.0 in the bath. The osmolarity in both solutions was adjusted to 420 mOsm by the addition of D-sorbitol.

Different concentrations of the flavonoids Naringenin, Naringin, Apigenin and Chrysin (from Sigma-Aldrich) were reached by dilution from DMSO stock solutions. Both stock solution and final solutions were freshly prepared just before the experiments to reduce the possibility of degradation due to exposure to light.

Polyunsaturated fatty acids (PUFAs): Arachidonic Acid (AA: 5,8,11,14-all-cis-eicosatetraenoic acid) and Docosahexaenoic acid (DHA: 4,7,10,13,16,19-all-cis-docosahexa-enoic acid) were purchased from Sigma-Aldrich. Pure fatty acids were dissolved in 99.5% ethanol to a concentration of 100 mM and stored at −20°C until usage. Right before experiments, stock solutions were diluted in pure control solution.

**Does DMSO and Ethanol affect the SV currents?**

In order to test if dimethyl sulfoxide (DMSO, stock solutions of Naringenin were prepared in DMSO) has any effect on SV currents, we performed experiments by adding DMSO at both cytosolic and luminal side. No significant effects were found when up to
0.2% (v/v) of DMSO was used (data not shown).
In order to test if Ethanol (stock solutions of PUFA were prepared in ethanol) has any effect on SV currents, we performed experiments by adding ethanol at cytosolic side. No significant effects were found when 0.9% (v/v) of ethanol was used (data not shown).
Results
Chapter 3.1. **Fluorescence imaging and flavonoid localization**

It is generally believed that flavonoids are synthesized on the multienzyme complexes at the cytoplasmic surface of the endoplasmic reticulum; then they are transported to different cell locations. However, the location of the glycosylation process is not well defined, so far. Therefore, before investigating the effects of the flavonoid Naringenin on the SV channel, we wanted to verify if non-glycosylated Naringenin is present in the cytoplasm. To this purpose, UDP-glucosyl transferase gene, which glycosylates Naringenin into Naringin, was isolated. *Arabidopsis thaliana* UDP-glucoronosyl/UDP-glucosyl transferase family protein (AT1G06000) (AtGT) was amplified using genomic DNA as a template, digested with NcoI enzyme, and cloned in frame with YFP as a fusion protein in the pGreen vector. The right orientation was confirmed by digestion and sequencing. Before studying the subcellular localization with this clone, western blot analysis was performed to confirm that AtGT-YFP is expressed as a fusion protein.

![Cloning strategy of AtGT gene in the pGreen Vector](image)

**Fig. 3.0.** Cloning strategy of AtGT gene in the pGreen Vector. (A) Multiple cloning sites of pGreen vector. (B) Vector map of pGreen 0029 vector.
Western Blot

To confirm the expression of fusion protein AtGT-YFP, western blot analysis was performed using total protein extract from infected tobacco leaves. Total proteins were extracted from the infected areas of the tobacco leaves and separated by 12% (w/v) SDS–PAGE, transferred to a nitrocellulose membrane and hybridised with a polyclonal antibody raised against YFP. The blot was then hybridised with an alkaline phosphate conjugated secondary antibody and the detection was performed with the NBT/BCIP colorimetric assay. This result confirms that the AtGT-YFP is expressed as a fusion protein of 79 kDa.

Fig. 3.1. Western blot analysis of AtGT-YFP fusion protein. Total extracted proteins (1, 2 and M are 3, 6 µg and protein marker respectively) were separated by 12% (w/v) SDS–PAGE, transferred to a nitrocellulose membrane and hybridized with a polyclonal antibody raised against YFP. The blot was then hybridized with an alkaline phosphate conjugated secondary antibody and the detection was performed with the NBT/BCIP colorimetric assay. Black arrow indicates the size of the fusion protein.
Subcellular localization of ATGT-YFP protein

To study the subcellular localization of AtGT-YFP, two experimental approaches were used. First approach was based on PEG transformation of Arabidopsis mesophyll protoplasts with pGreen AtGT-YFP vector; the second was based on agroinfiltration of tobacco leaves. YFP-dependent fluorescence was analysed after 24 hours in case of Arabidopsis mesophyll protoplasts and after 6 days after agroinfiltration of tobacco leaves.

A. Arabidopsis mesophyll protoplasts

B. Tobacco leaves

Fig. 3.2. Sub cellular localization of AtGT-YFP protein. (A) In Arabidopsis mesophyll protoplasts it was observed the expression of fusion protein of AtGT-YFP in the cytoplasm (left panel), chlorophyll (centre panel) and the merge of the two (right panel). (B) Transient expression of AtGT-YFP in tobacco leaves showing the expression of AtGT-YFP in the cytoplasm (left panel) and in the nucleus (centre panel); cytoplasmic strands were also noticed (right panel).

The results obtained on Arabidopsis mesophyll protoplasts are illustrated in Fig. 3.2 A showing the expression of AtGT-YFP fusion protein in the cytoplasm (left panel), of
chlorophyll (centre panel) and the merge of the two signals (right panel). Similar results were obtained in the confocal imaging of agro-infiltrated tobacco leaves as shown in Fig. 3.2 B where the expression of AtGT-YFP is mainly observed in the cytoplasm (left panel) and in the nucleus (centre panel); cytoplasmic strands are also clearly visible (see right panel).

These results indicate that the enzyme that glycosilates Naringenin into Naringin is present in the cytoplasm, thus emphasising that a certain concentration of free Naringenin should be present in the cytoplasm before its glycosylation.
Ch. 3.2. Macroscopic Slow Vacuolar currents

Typical voltage-dependent Slow activated Vacuolar (SV) currents, recorded in the tonoplast from Arabidopsis mesophyll cells in excised cytosolic side-out patch configuration, are shown in Fig. 3.3 A. These currents are mediated by the SV channel and are exclusively activated at positive tonoplast voltages (Bertl et al. 1992). These outward rectifying currents mainly represent the efflux of K$^+$ from the cytosol to the interior of the vacuole; here they are recorded in asymmetrical KCl, i.e KCl$_{\text{vac}}$=100 mM and KCl$_{\text{cyt}}$=200mM. The asymmetric current-voltage characteristics displayed in Fig. 3.3 B illustrates the channel activation only at positive membrane potentials while almost no current was detected at negative membrane potentials.

![Fig. 3.3. Macroscopic Slow Vacuolar currents recorded in a tonoplast from Arabidopsis mesophyll cells in excised cytosolic side-out patch configuration. (A) SV currents elicited by a series of voltage steps ranging from $-80$ mV to $+100$ in $+20$ mV steps. Solutions used were standard control solution 1; 200 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM dithiothreitol (DTT), and 10 mM HEPES/Tris, pH 7.5 in the bath. Holding potential $-50$ mV, tail potential $-50$ mV. (B) SV current-voltage characteristics of the traces shown in panel A. The I-V characteristics is constructed by plotting the average value of the currents recorded during the last 50 ms (delimited by vertical bars) at each applied voltage. Inset: schematic representation of the cation fluxes. The dashed circles represent the cell that in vivo surrounds the vacuole. Positive currents represent cations entering the vacuole and therefore leaving the cell.](image-url)
Therefore the SV channel encoded by TPC1 (see chapter 1.2.2) is a strongly outward rectifying channel that, in normal working conditions, only conducts positive currents at positive trans tonoplast potentials. The positive currents represent cationic charges flowing out of the cell into the vacuole (see Bertl et al. (1992) for the convention of the current).

It is interesting to observe that the kinetics of SV current activation may significantly change from one experiment to another. The reason for this variability (see Fig. 3.4) is not known so far and may depend on a series of different parameters including the membrane composition and the state of the plant or the tissue originating the vacuole.

**Fig. 3.4.** Macroscopic Slow Vacuolar currents recorded in two different carrot root vacuoles under identical working conditions. A stimulation protocol (see Fig. 2.6 for explanation) B and C panels show SV currents elicited by a series of voltage steps ranging from −80 mV to +120 in +20 mV steps. Tail voltage -50 mV, Holding voltage -50 mV. Solutions used were standard control solution 3; 200 mM KCl, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), and 10 mM HEPES/Tris, pH 7.5 in the bath.
Ch. 3.3. **Inhibition of the SV currents by Naringenin added at the cytosolic side**

Looking for compounds able to modulate the SV currents we challenged this channel with Naringenin, a flavonoid playing an important role in plant cells. **Fig. 3.5** illustrates the results induced by Naringenin on the SV currents. The traces represent the typical current response of the SV channel stimulated by a voltage step to +80 mV. When the bath solution was replaced with an identical solution containing 1000 µM Naringenin, we observed a significant inhibition in the SV currents of vacuoles obtained from either (A) *Arabidopsis* mesophyll protoplasts or (B) carrot roots. In both cases when the Naringenin-enriched solution was replaced with a Naringenin-free bath solution, the original currents were completely restored.

**Fig. 3.5.** Effects of the flavonoid Naringenin on Arabidopsis and carrot SV currents. (A) SV currents in response to voltage stimulation at V= +80 mV in control conditions, in the presence of Naringenin (1000 µM) and after washout (recovery) in vacuoles from Arabidopsis mesophyll protoplasts and B in vacuoles from carrot roots in identical working conditions as in A. Holding voltage -50 mV, tail voltage -50 mV. Solutions: standard control solution 1 (see materials and methods).

Presumably the current inhibition mediated by Naringenin is a characteristic of the SV channel that does not depend on the tissue and the plant species where this channel is expressed. More experiments on vacuoles from other plants and tissues are needed to further support this preliminary evidence.
Fig. 3.6. Time course of the inhibition induced by Nar, measured by the fast perfusion procedure (see materials and methods). Vacuoles were isolated from carrot roots. Panel A shows the inhibition and the recovery of two typical SV currents (blue and green traces) when they are perfused with a solution supplemented with 1000 µM Nar (indicated by the top black line) in identical working conditions. Continuous voltage at V= +80 mV. The grey trace superimposed to the green trace represents the time needed by the fast perfusion setup to change the solution bathing the vacuole. This was obtained in test experiments perfusing the patch pipette with a diluted solution (1:10 by water) and returning back to standard control solution 1. The times needed for the inhibition and recovery of the current at different voltages are plotted in panels B (on) and C (off), respectively. Applied voltages from +40 to +120 mV in +20 mV steps. Solutions used were standard control solution 1 (see materials and methods).
connect different points from the same vacuole. Different experiments are represented by diverse symbols.

In order to clarify the mechanism of interaction of Naringenin with the membrane phase, we also measured the time course of inhibition induced by Nar by the fast perfusion procedure applied to vacuoles isolated from carrot roots under continuous voltages. The results are reported in the Fig. 3.6. Panel A shows typical SV currents at $V=+80$ mV and the inhibition and recovery of these currents while perfusing the membrane patch with a solution supplemented with 1000 µM Naringenin (black line on the top) in two different experiments (blue and green traces) under identical working conditions. Clearly, in some vacuoles the SV currents displayed a faster inhibition and recovery while others showed a slow time course (compare top and bottom panels of Fig. 3.6 A). Notably, the variability of the kinetics of current inhibition and recovery induced by Nar spans almost three orders of magnitudes (Fig. 3.6 B).

Interestingly, the time course of current inhibition was faster compared to the recovery (compare B and C in Fig. 3.6) in the order of a few seconds. These data suggests that the absorption/desorption of Nar from the membrane phase changes significantly from vacuole to vacuole as it possibly depends on a series of parameters controlling the membrane chemico-physical characteristics.
Ch. 3.4. Quantitative analysis of SV channel inhibition by Naringenin

In order to gain a better understanding of the effects induced by Naringenin on the SV currents, we performed dose-response experiments in the concentration range from 100 µM to 5 mM Naringenin in carrot root vacuoles. A dose-dependent decrease of the SV channel activity was measured. For example at V= +80 mV, on the addition of [Nar] concentrations as small as 150 µM, the residual SV current (I_{Nar}/I_{control}) was about 0.2, while 5 mM Naringenin decreased the SV currents to 5% of the control. This modulation of the current was fully reversible. These results are illustrated in Fig. 3.7, where I_{Nar}/I_{control} is plotted as a function of Naringenin concentration. A curve fitting with the Hill equation (see eq. 2.1):

\[
I_{Nar}/I_{control} = 1/(1+([Nar]/K_h)^n)
\]

gave a half inhibition concentration of (0.44 ± 0.03) mM. In eq. 2.1 [Nar] is the Naringenin concentration, K_h and n (=1.4 ± 0.1, in Fig. 3.7) are the Hill half inhibition constant and Hill coefficient, respectively.

Fig. 3.7. Quantitative analysis of SV current inhibition by Naringenin: I_{Nar}/I_{control} plotted as a function of Naringenin concentrations. We obtained a half inhibition constant K_h = (0.44 ± 0.03) mM with a Hill equation where n=1.4 ± 0.1. These data values were derived normalizing the steady state current measured in the presence of Nar to the steady state level obtained in control conditions, i.e. before the addition of Nar.
Fig. 3.8. Quantitative analysis of SV current inhibition by Naringenin: $G_{\text{norm}}$ plotted as a function of Naringenin concentrations. A) Normalized conductance, with respect to control conditions plotted vs Nar concentrations in the voltage range from +30 to +120 mV; note that only three voltages are represented for clarity reasons. For all the potentials under investigation a fit curve was performed and the Hill inhibition constant ($K_h$) and Hill coefficient (n) were obtained. B) $K_h$ values, derived from the Hill fit shown in panel A are plotted against the applied membrane voltages. C) Hill coefficients (n) are plotted against the applied membrane voltages. The value of n is >1 at all the investigated voltages.
Similar results were obtained when $G_{\text{norm}}$, the conductance normalized with respect to the control conditions in the absence of Nar was plotted as a function of Nar concentration using the tail currents*. The results obtained in these experiments are illustrated in Fig. 3.8 panel A. Panel B shows $K_h$ values, derived from the Hill fit shown in panel A, plotted against the applied membrane voltages. With this fit we obtained Hill coefficients $n>1$ at all the investigated voltages (Fig. 3.8 C).

These results constitute an indirect indication of a specific interaction between Naringenin and the SV channel: indeed a positive cooperation between Nar and the channel suggests that more than one Nar molecule is necessary to inhibit the channel and the binding of one flavonoid molecule further facilitates Nar binding to the protein.

* Note that data in Fig. 3.8 were retrieved from voltage-dependence analysis illustrated in Fig. 3.9.
Ch. 3.5. Voltage dependence characteristics of the SV channel in the presence of cytosolic Naringenin

A further insight into the SV channel properties modified by Naringenin can be obtained investigating the voltage-dependence of the channel in the presence and absence of this flavonoid. **Fig 3.9 A** illustrates the currents mediated by the SV channel of carrot root vacuoles at different voltages ranging from -80 mV to +120 mV in +10 mV steps, in control conditions (left traces), in the presence of 500 µM Naringenin (centre) and in recovery conditions (right traces) (note that only one out of two traces are reported in **Fig. 3.9 A** for clarity reasons). Indeed, macroscopic currents were strongly reduced when 500 µM Naringenin was added to the bath solution, while, upon removal of Naringenin, current inhibition was fully reversed. In **Fig. 3.9 B** normalized conductances derived from instantaneous tail currents are plotted as a function of the main voltage pulse (for definition of tail potential see **Fig. 2.6** and **Fig. 3.4**) and fitted with a single Boltzmann function. Data analysis confirmed that the addition of Nar affects the voltage dependence of the channel shifting the activation to more positive voltages, increasing s and decreasing the maximum conductance. These parameters are reported in **table 1**. **Fig. 3.9 C** shows the time required for half-activation ($t_{1/2\,\text{norm}}$) of the current at different membrane potentials. The values obtained in control/recovery conditions are comparable to those obtained in the presence of Nar. Instead, the time constant of deactivation ($\tau_{\text{norm}}$) derived from tails currents in the presence of Nar are faster compared to the values derived in control/recovery conditions.

These data indicate that Nar, determines a modification of the energy needed for activation by the voltage sensor of the channel, which, in the presence of the flavonoid, opens at much more positive membrane potentials. So far is not clear whether this process occurs via a direct or indirect interaction of the flavonoid with the channel.
Fig. 3.9. Inhibition of the SV currents by Naringenin is voltage dependent. (A) Macroscopic SV currents, elicited by a series of voltage steps ranging from -80 to +120 mV in +10 mV steps in control (left panel), in the presence of 500 µM Naringenin (centre panel) and after recovery (right panel) (note that only one out of two traces was reported for clarity reasons). Holding potential -50 mV, tail potential -50 mV. Solutions used were standard control solution 3 (see materials and methods). (B) Normalized conductances derived from instantaneous tail currents were plotted as a function of the main pulse and fitted with a single Boltzmann function (see materials and methods). Note that normalization is done with respect to the control. Data points represent the mean of nine experiments in control and at least three experiments at various concentrations of Nar; error bars represent SEM. (C) Normalized time required for half-activation of the current (t½ norm) is plotted against the applied membrane potential. Data points represent mean values (± SEM) determined in control conditions (open circles; n = 9), in the presence of 300 µM Nar (closed circles; n = 3) and 500 µM Nar (closed triangles; n = 3). Data points are normalized to the value of control at V = + 60 mV. (D) Normalized time constants of current deactivation, τ norm are plotted against the tail potential. Data points represent mean values (± SEM) of τ norm determined in control conditions (open circles; n = 9) and in the presence of 300 µM Nar (closed circles; n = 3) and 500 µM Nar (closed triangles; n = 3). Data points are normalized to the value of control at V = - 60 mV.
Table 1

<table>
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<th></th>
<th>$V_{1/2}$ (mV ± SEM)</th>
<th>(n)</th>
<th>$s$ (mV ± SEM)</th>
<th>$G_{\text{Max}}(\text{Nar})/G_{\text{Max}}(\text{control})$</th>
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<tr>
<td>Control</td>
<td>18 ± 4 (9)</td>
<td></td>
<td>19 ± 1</td>
<td>1</td>
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<tr>
<td>Nar= 300 µM</td>
<td>33 ± 7 (3)</td>
<td></td>
<td>27 ± 4</td>
<td>0.70 ± 0.07</td>
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<tr>
<td>Nar= 500 µM</td>
<td>60 ± 11 (3)</td>
<td></td>
<td>36 ± 4</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>Nar= 1000 µM</td>
<td>62 ± 7 (3)</td>
<td></td>
<td>39 ± 1</td>
<td>0.40 ± 0.07</td>
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**Table 1:** Half-activation potentials, $V_{1/2}$, $s (=RT/zF)$ and ratios of the maximum conductances in the presence of and absence of Naringenin $G_{\text{Max}}(\text{Nar})/G_{\text{Max}}(\text{control})$, derived from eq. 2.4, are reported in the table as function of Nar concentration. Note that an increase of the “s” means a decrease of the apparent gating charge z.

Nevertheless a comparison of the activation and deactivation kinetics suggests that the effects mediated by Nar are not due to a simple shift of the activation characteristics of the channel as the activation and deactivation kinetics are modified in a different manner. This is incompatible with a single shift of the activation characteristics of the channel to be ascribed, for example, to a modification of the surface charges at the membrane/protein-water interface.
Ch. 3.6. **Naringenin effects on the SV single channel conductance**

To investigate whether Naringenin binds within the pore of the SV channel and affects the single channel conductance, we performed single channel experiments on vacuoles from carrot roots with 500 µM Nar. Two voltage protocols were used. In the first approach the current response to continuous voltage stimulation, e.g. at $V = -70$ mV (like in the Fig. 3.10) was recorded. At this voltage the open probability of the SV channel is very low and consequently the single channel signals can be recorded in more appropriate conditions i.e. the recordings contain one or a few channels. Under these conditions, control solution (standard control solution 4) was replaced with an identical solution supplemented with 500 µM Nar (Fig. 3.10).

![Graph showing single channel current transitions in control conditions, in the presence of 500 µM Nar and after recovery.](image)

**Fig. 3.10.** Naringenin does not change the amplitude of the conductance of the SV single channel. Single channel current transitions in control conditions, in the presence of 500 µM Nar and after recovery. C, O₁ and O₂ represent the closed state, one channel and two channels open respectively. Solutions used were, 100 mM KCl, 20 mM HEPES/5 mM KOH, pH 7.0 and 100 µM fura-2 in the pipette; 100 mM KCl, 2 mM CaCl₂, 2 mM dithiothreitol (DTT), and 20 mM HEPES/5 mM KOH, pH 7.0 in the bath (standard control solution 4). Applied potential $V = -70$ mV.
**Fig. 3.10** illustrates the single channel recordings in the absence and in the presence of 500 µM Nar. Top panel shows the single channel openings at V= -70 mV. When we replaced the control solution with an identical solution supplemented with 500 µM Nar the single channel conductance did not change appreciably (compare C and O₁ of control and Nar), while the channel remained closed most of the time and the mean open time decreased drastically. The original firing pattern of the channel was completely restored when we replaced the Nar supplemented solution with the control solution (see recovery in **Fig. 3.10**).

![Graph](image)

**Fig. 3.11.** A) Single channel current–voltage relationships obtained after the application of a fast voltage-ramp in control conditions (black trace) superimposed to data points (filled circles) obtained under continuous voltage stimulation in the presence of 500 µM Nar. Cytosolic side-out excised patch from the vacuoles of carrot roots. Solutions used were, 100 mM KCl, 20 mM HEPES/5 mM KOH, pH 7.0 and 100 µM fura-2 in the pipette; 100 mM KCl, 2 mM CaCl₂, 2 mM dithiothreitol (DTT), and 20 mM HEPES/5 mM KOH, pH 7.0 in the bath (standard control solution 4). B) Voltage protocol applied to the membrane patch to record single channel characteristics by means of a voltage-ramp. Voltage frequency= 2.5 V/S.

The second protocol was based on fast voltage ramps in order to get access to the conductance at far positive voltages where single channels cannot be resolved because of high number of channels that open simultaneously. If the ramp is sufficiently fast, one can span the entire voltage range of interest starting from negative voltage, completing the cycle before more channel superimpose to the channel signals recorded at negative voltages. In **Fig 3.11** the single channel conductance obtained from voltage ramps is
represented as a black trace superimposed to the single channel data (filled circles) obtained from the continuous voltage protocol in the presence of 500 µM Nar. The data (n=204 mean ± SD) from Fig. 3.10 are also represented at –70 mV.

These data provide further support to the results illustrated in Fig. 3.10 and guarantee that the single channel conductance does not change in the presence or absence of Nar in the whole range of the investigated voltages. Moreover ramp signals illustrated in Fig. 3.11 refer to one single protein unit. While single channel recordings obtained under continuous voltage protocols might be representative of successive openings of one or more single protein units and therefore request a time consuming statistical analysis.

Note the smaller single channel conductances at positive voltages with respect to the negative voltages. This is due to the working conditions (i.e. high cytosolic, low vacuolar calcium concentrations). The reversal voltage is about 0 mV in accordance with symmetrical KCl solutions that give a V_{Nernst} = 0 mV.
Ch. 3.7. **Simultaneous Fluorescence measurements and patch-clamp recordings**

The SV channel is not very selective to different cations. Indeed, it has been shown that it is permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions (see Chapter 1.2.2). In order to verify whether Nar affects both K\(^+\) and Ca\(^{2+}\) conductances, we performed experiments by combining the patch-clamp technique with fluorescence measurements using the fluorophore fura-2 on *Arabidopsis thaliana* mesophyll and carrot root vacuoles. Permeation of calcium was demonstrated by conductance measurements (Pottosin et al. 2001) and reversal potential determination in the presence of varying concentration ratios of potassium and calcium: (Allen and Sanders 1996).

![Fig. 3.12. Calcium permeation through Slow Vacuolar channels isolated from carrot roots. Simultaneous fura-2 fluorescence and macroscopic current recordings on a cytosolic side-out tonoplast patch. Fura-2 signals resulting from alternate excitation at 340 and 380 nm are shown. B) After reaching the cytosolic side-out excised patch configuration, 10-s voltage pulses were applied according to the voltage scheme. The respective current responses are shown in the panel C. Solutions used were standard control solution 4 (see materials and methods).](image-url)
The experimental approach was based on the idea by Neher (1995) to simultaneously record whole cell currents (using the patch-clamp technique) and the fluorescence signal of the calcium indicator dye fura-2. Unless otherwise indicated, our experiments were conducted in the cytosolic-side-out excised patch configuration; the fluorescence signal was collected focusing the photomultiplier to the tip of the pipette loaded with the fluorescent dye. In these working conditions fluorescence signals (Fig. 3.12 A) and Slow Vacuolar currents (Fig. 3.12 C) were recorded simultaneously upon voltage stimulation (Fig. 3.12 B). Stimulation of SV currents coincided with characteristic changes in fluorescence in vacuoles from carrot roots. The emission upon excitation at 380 nm decreased, while the signal at 340 nm increased increasing the membrane potential (Fig. 3.12 A). This behavior is indicative of calcium permeation through the channel and subsequent binding of Ca$^{2+}$ to the fluorescent dye (Gryniewicz et al. 1985).

To verify whether fluorescence is strictly linked to calcium transport mediated by SV channel, experiments were conducted in vacuoles from Arabidopsis thaliana wild-type and tpc1 mutant plants that lack SV channel activity (Peiter et al. 2005). Similar to carrot root vacuoles, voltage stimulation of a cytosolic-side-out membrane patch derived from an Arabidopsis wild-type vacuole elicited both macroscopic SV currents and simultaneous fluorescence changes; instead, vacuoles from tpc1 knockout plants, besides lacking SV channel activity, did not show any variation of the fluorescence signals, neither upon prolonged voltage stimulation (Fig. 3.13). These results confirmed that the changes in fura-2 fluorescence amplitudes indeed reflected Ca$^{2+}$ currents through SV channels. Details on this approach and the corresponding results are reported in Gradogna et al. (2009).
Fig. 3.13. The fura-2 fluorescence response is absent in Arabidopsis tpc1 knockout vacuoles. Changes in fura-2 fluorescence were recorded upon voltage stimulation of a cytosolic side-out tonoplast patch derived from an Arabidopsis thaliana Col-0 vacuole (wild type, left panels). No fluorescence response was present when patches from tpc1-2 knockout vacuoles were tested (right panel). The respective current responses are shown in the lower panels. Vacuoles were isolated from leaf mesophyll protoplasts. Solution used was standard control solution 4 (see materials and methods).

In order to investigate the effects of flavonoids in our working conditions, control solution was replaced by solutions containing different concentrations of Nar. Fig. 3.14 illustrates the results obtained in such patch-clamp experiments performed on carrot root vacuoles. Fluorescence signals observed at 380 (Fig. 3.14 A upper panel) and 340 nm (Fig. 3.14 A middle panel) are shown together with the applied membrane potential (protocol, Fig. 3.14 A lower panel). When 200 µM Naringenin was added to the bath solution (central panel in A) we could observe a reduction in fluorescence (middle panel A) with respect to the control condition (left panel A). This effect was fully reversible, as, when we replaced 200 µM Naringenin solution with the bath solution; fluorescence was completely restored (right panel A).
Fig. 3.14. Patch-clamp recordings with fluorescence measurements on carrot root vacuoles. (A) Changes in fura-2 fluorescence in control conditions were elicited by a voltage step to +80 mV from a holding potential of -80 mV (see lower panel illustrating the applied voltage). In the presence of 200 µM Naringenin fluorescence signal was reduced. The effect was fully reversible after washout of Naringenin (recovery). (B) Changes in fura-2 fluorescence in control conditions were elicited by a voltage step to +80 mV from a holding potential of -80 mV (see lower panel). In the presence of 1000 µM Naringenin, fluorescence signal was almost absent. The effect was fully reversible after washout of Naringenin (recovery). (C) SV currents responses upon voltage
stimulation in control and in the presence of 200 µM Naringenin. (D) SV currents responses upon voltage stimulation in control and in the presence of 1000 µM Naringenin. Solutions used were standard control solution 4 (see materials and methods).

The results obtained with 1000 µM Naringenin are illustrated in Fig. 3.14 B. Fluorescence signal observed at 380 (upper panel) and 340 nm (middle panel B) are shown together with the applied membrane potentials (lower panel B). On the addition of 1000 µM Naringenin, the fluorescence signal was completely abolished. Current records reported in panels 3.14 C and D integrate the fluorescence experiments illustrated in panel 3.14 A and B. Furthermore Fig. 3.14 C illustrates SV channel current response on prolonged voltage stimulation in control and 200 µM Naringenin. Finally Fig. 3.14 D illustrates SV channel current response on prolonged voltage stimulation in control and in the presence of 1000 µM Naringenin.

The substantial equivalence of electrophysiological and fluorescence signals definitely demonstrate that flavonoid Naringenin affects both K⁺ and Ca²⁺ conductances.

As illustrated in the Discussion section this observation provides a substantial evidence of the fact that Naringenin does not directly bind within the pore or in the proximity of the ionic pathway.
Ch. 3.8. **pH and DTT effects on the modulation of the SV channel by Naringenin**

It has been reported that, owing to its hydrophobic nature Nar can partition into the hydrophobic core of the lipid bilayer (van Dijk et al. 2000) modulating the membrane fluidity (Arora et al. 2000). It has also been demonstrated that the flavonoid Quercetin (QCT) can penetrate into the lipid bilayer to different extents at different pHs. This depends on the neutral form and liposolubility of QCT at acidic pHs and to its deprotonated form at more alkaline pHs (Movileanu et al. 2000). An increasing value in the pH of the bath solution also promotes a more hydrophobic character of Naringenin (van Dijk et al. 2000). On this basis we decided to verify whether a variation of the pH has any effect of the inhibition of the SV channel; therefore we changed the pH of external solution from 7.5 to 6.5.

Under these conditions we challenged the SV currents with 500 µM Nar.

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**Fig 3.15.** pH and DTT modify the inhibition of SV currents by Naringenin. (A) The graph represents the residual SV currents ($I_\text{Nar}/I_\text{control}$) when 500 µM Nar was added to the bath at pH 7.5 and pH 6.5 (n=3). Solutions used were standard solution 1 (see materials and methods). (B) Residual SV currents ($I_\text{Nar}/I_\text{control}$) when DTT was increased from no DTT to 1 mM DTT and 5 mM DTT in the presence of 500 µM Nar (n=3). Error bars indicate SEM.
Panel 3.15 A represents the residual SV currents ($I_{Nar}/I_{control}$) when 500 µM Nar was added at pH 7.5 and pH 6.5 (n=3). Our results demonstrate that a decrease of the pH determines an increase of the inhibition of the SV currents by Nar; thus it supports the possibility that Nar interacts with the SV channel through the phospholipid bilayer.

It has been reported that DTT increases the open probability of the SV channels, possibly interacting with sulphydryl groups of cysteine residues located at the cytoplasmic side of the channel (Carpaneto et al. 1999).

As the control standard solution used in normal conditions contains 1 mM DTT (that prevents SV current rundown) and flavonoids have well known antioxidant properties, we decided that it was worthwhile to investigate the effects induced by different DTT concentration on SV currents in the presence of Naringenin. **Fig. 3.15 B** illustrates the results obtained varying DTT concentration. The graph represents the residual SV currents ($I_{Nar}/I_{control}$) when DTT was absent or in the presence of 1 mM DTT or 5 mM DTT and 500 µM Nar.

Although these experiments produced data with no significant differences, one can observe a general trend where the inhibition of the SV currents induced by Nar decreases increasing DTT (n=3).

More experiments are needed, in order to understand the effects of reducing agents that may compete or interact with other antioxidants, changing their modulation of the SV channel properties; however this detailed investigation goes beyond the present scope of this thesis. Therefore these preliminary studies on the role of DTT on Nar action are preliminary to a detailed characterization of the effects of flavonoids and PUFAs (see Ch. 3.13) in diverse redox conditions (Angelova et al. 2009).
Ch. 3.9. **Effects of vacuolar Naringenin on the SV channel**

We also investigated the effects induced on the SV channel by Naringenin added at the luminal side, in comparison with those induced by Nar at the cytosolic side. **Fig. 3.16 A** illustrates SV currents in response to a voltage step to +80 mV in control, in the presence of 300 µM Naringenin and in recovery conditions in vacuoles from *Arabidopsis* mesophyll cells. The average residual SV current ($I_{\text{Nar}}/I_{\text{control}}$) was $0.55 \pm 0.03$ SEM (n=5). **Fig. 3.16 B** illustrates similar experiments performed on a vacuole from carrot roots using 1000 µM Nar. Macroscopic currents were strongly reduced when Naringenin was added to the bath solution also in these working conditions; upon removal of Naringenin, current inhibition was fully reversed.

**Fig. 3.16. Luminal Naringenin decreased the SV current.** Excised luminal side-out patch showing voltage dependent outward currents mediated by the SV channel at positive membrane potential. (A) SV current response to voltage stimulation at $V= +80$ mV (control), in the presence of Naringenin (300 µM) and after washout (recovery). Vacuoles were isolated from Arabidopsis thaliana mesophyll cells. Solutions used were standard solution 5 (see materials and methods). (B) SV current response to voltage stimulation at $V= +80$ mV (control), in the presence of Naringenin (1000 µM) and after washout (recovery). Holding voltage -50 mV, tail voltage -50 mV. Vacuoles were isolated from carrot roots. Solutions used were standard solution 6 (see materials and methods).

These data demonstrate that Nar presents similar modulation characteristics of the SV channel activity at both sides of the membrane. Therefore, presumably the binding site is not located at the membrane water interface (either at the cytosolic or the luminal side), but it is readily accessible by both membrane sides. These considerations lead us to investigate whether the glycosylated form of Naringenin has also similar consequences on the channel.
Ch. 3.10. **Naringin does not inhibit the SV currents**

As the glycosylated form of Naringenin, Naringin, has different hydrophobicity characteristics with respect to the non-glycosylated form, we investigated whether Naringin also has the ability to change the transport properties of the SV channel.

![Naringenin and Naringin](image)

**Fig. 3.17. Structure of Naringenin and Naringin**

**Fig. 3.18 A** illustrates the consequences of the addition of Naringin at the cytosolic side of the vacuole: it is shown the SV current response to voltage stimulation at $V= +80$ mV in control conditions, in the presence of 1000 µM Naringin and after the recovery. In vacuoles isolated from *Arabidopsis thaliana* mesophyll cells, Naringin displayed a negligible inhibition of the SV currents ($n=3$). **Fig. 3.18 B** illustrates the effects of Naringin at the vacuolar side; it is shown the SV channel current response on voltage stimulation at $+80$ mV in control, in the presence of 1000 µM Naringin and in recovery conditions. Also in these conditions Naringin did not show any modulation of the SV currents ($n=5$).
Fig. 3.18. The glycosylated flavonoid Naringin only slightly inhibits the SV currents in vacuoles isolated from Arabidopsis thaliana mesophyll cells. (A) SV channel response to voltage stimulation at V= +80 mV (control), in the presence of cytosolic Naringin (1000 µM) and after washout (recovery). Holding potential -50 mV, tail potential -50 mV. Solutions were 200 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM MES/Tris, pH 5.5 in the pipette; 100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), and 10 mM HEPES/Tris, pH 7.5 in the bath (standard control solution 1). (B) SV channel response to voltage stimulation at V= +80 mV (control), in the presence of vacuolar Naringin (1000 µM). Solutions were 100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), and 10 mM HEPES/Tris, pH 7.5 in the pipette; 200 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM MES/Tris, pH 5.5 in the bath solution (standard control solution 5).

In conclusion only the non-glycosylated form, Naringenin, does affect the channel activity. We hypothesize that the difference in the effects produced by Naringenin and Naringin depends on the higher hydrophobicity of the non-glycosylated flavonoid with respect to the glycosylated form that carries a relatively large hydrophilic sugar terminal attached to the Naringenin scaffold.
Ch. 3.11. Other cytosolic flavonoids also inhibit the SV currents

In order to investigate whether other flavonoids sharing structural similarities with Nar produce effects on the SV channel currents, we challenged a few vacuoles with the flavonoids Apigenin and Chrysin (Fig. 3.19).

![Naringenin](image1)

**Naringenin**

![Apigenin](image2)

**Apigenin**

![Chrysin](image3)

**Chrysin**

**Fig. 3.19. Structure of flavonoids used in this study.**

The results obtained in such experiments are illustrated in Fig. 3.20. Panel A shows the typical response of the SV channel after a voltage stimulation to $V = +80$ in the presence of 300 µM Nar and in recovery conditions. Panels B and C show the responses of SV channel (still at $V = +80$) in the presence of 300 µM Apigenin and Chrysin respectively as well as in recovery conditions. These results indicate that flavonoids, sharing structural similarities with Nar, determine a similar modulation of the SV currents.
Fig. 3.20. Other cytosolic flavonoids modulate the SV currents similarly to Nar. A shows the typical response of SV channel at \( V = +80 \) in control conditions and in the presence of 300 \( \mu M \) Nar. B and C show the responses of SV channel \( (V = +80) \) in the presence of 300 \( \mu M \) Apigenin and Chrysin. Holding potential -50 mV, tail potential -50 mV. All the experiments were performed on vacuoles from carrot roots. Solutions used were standard control solution 1 (see materials and methods).
Ch. 3.12. Effects of Naringenin on the SV channel from transparent testa mutant plants

Interestingly the seed coats of *Arabidopsis thaliana* transparent testa mutant plants are affected by a deficiency in the flavonoid biosynthetic pathway (Shirley et al. 1995; Buer and Muday 2004; Buer and Djordjevic 2009). In order to verify whether SV currents in these flavonoid deficient plants display different modulation by Nar, we performed some experiments on these plants. Two mutant plants were investigated, namely *tt4* and *tt5*, which lack the flavonoid biosynthetic pathway and Naringenin, respectively. As the *tt* mutant plants belong to the Landsberg erecta ecotype, these plants were also included in this study.

![Fig. 3.21](image)

**Fig. 3.21.** Cytosolic Naringenin inhibits SV currents from transparent testa mutant plants. **A** The SV channel response to voltage stimulation at *V*= +80 mV in control, in the presence of 1000 µM Naringenin and in recovery conditions in vacuoles from mesophyll cells of Landsberg erecta plants. **B** and **C** illustrate the results obtained in *tt4* and *tt5* mutant plants respectively; the same working conditions as in **A**. Holding potential -50 mV, tail potential -50 mV. Solutions used were standard control solution 1 (see materials and methods).
Fig. 3.21 illustrates the results obtained in such experiments. Panel A illustrates the SV channel response to voltage stimulation at +80 mV in control, in the presence of 1000 µM Naringenin and in recovery conditions in vacuoles from mesophyll cells of Landsberg erecta plants. B and C illustrate the results obtained in tt4 and tt5 mutant plants respectively in the same working conditions as in A.

We observed a reversible decrease in the activity of the currents when 1000 µM Nar was added to the cytosolic side, thus demonstrating that Nar displays similar modulation capability of the SV current also in vacuoles isolated from plants that lack flavonoids.
Ch. 3.13. Effects of polyunsaturated fatty acids on the SV currents

It has been reported that Naringenin can partition in the hydrophobic core of the lipid bilayer (van Dijk et al. 2000) modulating the membrane fluidity (Arora et al. 2000). It has also been demonstrated that flavonoids could penetrate into the lipid bilayer to different extents at different pHs. For example flavonoid Quercetin is deeply embedded in planar lipid bilayers at acidic pH (Movileanu et al. 2000) while, on the contrary, it interacts at the water-lipid interface at physiological pHs (Terao et al. 1994; Movileanu et al. 2000). Indeed our pH experimental data are consistent with these results (see Ch. 3.8), thus it supports the hypothesis that Nar interacts with the SV channel through the phospholipid bilayer. To verify this possibility, we investigated the interaction of the SV channel with polyunsaturated fatty acids (PUFAs). Indeed many studies are aimed at verifying modulation of mammalian ion channels by PUFAs. For review on this topic see Boland and Drzewiecki (2008).

Two PUFAs, namely Arachidonic Acid (AA: 5,8,11,14-all-cis-eicosa-4,7,10,13,16,19-all-cis-docosahexaenoic acid) and Docosahexaenoic acid (DHA: 4,7,10,13,16,19-all-cis-docosahexaenoic acid), were used in this study (Fig. 3.22).

![Arachidonic acid and Docosahexaenoic acid](image)

**Fig. 3.22. Structure of PUFA included in this study. Arachidonic Acid (AA) and Docosahexaenoic acid (DHA).**

These experiments, performed on vacuoles from carrot root vacuoles, are illustrated in Fig. 3.23. Top Panel in A shows the typical response of the SV channel to voltage steps at V= +80 in control conditions and in the presence of 1 µM AA. Middle and bottom panels show the inhibition of the currents on the addition of 3 µM AA and 10 µM AA, respectively. In all the cases original currents were restored when the AA-supplemented solutions were replaced with the control solution. Similar results were obtained when DHA (see panel B) was used in place of AA in the same experimental conditions as shown in Fig. 3.23 A.
Fig. 3.23. Cytosolic PUFAs inhibit the SV currents. (A) Top panel shows the SV current in control conditions, in the presence of 1 µM AA and after washout (recovery). Middle panel shows the SV channel response in control conditions, in the presence of 3 µM AA and after washout (recovery). Bottom panel shows the SV channel in control conditions, in the presence of 10 µM AA and after washout (recovery). (B) Top panel shows the SV current in control conditions, in the presence of 3 µM DHA and after washout (recovery). Bottom panel shows the SV channel response in control conditions, in the presence of 10 µM DHA. Vacuoles were isolated from carrot roots. Applied voltage $V = +80$ mV holding potential -$50$ mV, tail potential -$50$ mV. Solutions used were standard control solution 3 (see materials and methods).

Panel B top traces shows the typical responses of the SV channel to voltage stimulations at $V = +80$ in control conditions and in the presence of 3 µM DHA. The bottom panel B
shows the current inhibition mediated by 10 µM DHA. In both cases original currents were restored when the DHA solutions were replaced with control solution.

In conclusion our data strongly suggest that the SV channel is modulated by flavonoids but also by other compounds, such as PUFAs, that possibly interact with the protein through the lipid double layer. However, so far it is not clear whether these compounds modify the SV channel properties directly or changing the organization and fluidity of the bilayer.

More experiments are needed to clarify this point. For example capacitance measurements may provide indications on the presence of PUFA layers at the membrane-water interface.
Ch. 3.14. **Voltage dependence characteristics of the SV channel in the presence of cytosolic Arachidonic Acid**

A further insight into the channel properties modified by Arachidonic Acid can be obtained investigating the voltage-dependence of the channel in the presence and absence of AA. In **Fig 3.24 A** the normalized conductances obtained from instantaneous tail currents were plotted as a function of the main voltage pulse and fitted with a single Boltzmann function.

Data analysis confirmed that also the addition of AA affects the voltage dependence of the channel shifting the activation to more positive voltages, increasing “s” and decreasing the maximum conductance. The values of these parameters in different working conditions (i.e. in the absence and in the presence of different AA concentrations) are illustrated in **table 2**.

**Fig. 3.24 B** shows the time required for half-activation ($t_{\text{half norm}}$) of the current at different membrane potentials. The values obtained in the presence of AA are faster compared to the values derived in control/recovery conditions. **Fig. 3.24 C** shows that the time constant of deactivation, derived from conductances in the presence of AA, is faster compared to the values derived in control conditions. AA possibly interacts with the voltage sensor of the channel or changes the membrane properties in a manner that determines the activation of the SV channel at much more positive membrane potentials.
Fig. 3.24. The inhibition of the SV current induced by Arachidonic Acid is voltage dependent. (A) Normalized instantaneous tail currents were plotted as a function of the activating potential and fitted with a single Boltzmann function. Data points represent the mean of eight experiments in control and three experiments from different vacuoles at various concentrations of AA as indicated. Error bars represent SEM. (B) Normalized half-time of the current activation is plotted against the applied potential. Data points represent mean values (± SEM) determined in control conditions (open circles; \( n = 8 \)), in the presence of 1 µM AA (closed red circles; \( n = 3 \)) and 3 µM AA (closed gray triangles; \( n = 3 \)). Data points are normalized to the values obtained at \( V = +60 \) mV. C) Time constants of current deactivation \( \tau \) are plotted against the tail potential. Data points represent mean values (± SEM) of \( \tau \) determined in control conditions (open circles; \( n = 8 \)) and in the presence of 1 µM (closed red circles; \( n = 3 \)) and 10 µM AA (closed gray triangles; \( n = 3 \)).
### Table 2

<table>
<thead>
<tr>
<th></th>
<th>$V_{1/2}$ (mV ± SEM)</th>
<th>$s$ (mV ± SEM)</th>
<th>$G_{\text{Max}}(\text{AA})/G_{\text{Max}}(\text{control})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39 ± 5 (8)</td>
<td>21 ± 1</td>
<td>1</td>
</tr>
<tr>
<td>AA= 1 µM</td>
<td>58 ± 2 (3)</td>
<td>32 ± 2</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td>AA= 3 µM</td>
<td>48 ± 14 (3)</td>
<td>36 ± 4</td>
<td>0.51 ± 0.13</td>
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**Table 2**: Half-activation potentials, $V_{1/2}$, $s$ (=RT/zF) and the ratios of the maximum conductances $G_{\text{Max}}(\text{AA})/G_{\text{Max}}(\text{control})$, in the presence and absence of AA derived from eq. 2.4, are reported in the table as a function of AA concentration. Note that an increase of the “s” implies a decrease of the apparent gating charge, z.

However, differently from what observed on the addition of Naringenin, a comparison of the activation and deactivation kinetics shows that AA slows down both the activation and deactivation times. Nevertheless, also in this case (AA mediated inhibition) the slower kinetics of current activation is incompatible with a shift of the activation characteristics due to surface charge modifications at the membrane-water interface.
Ch. 3.15. **Quantitative analysis of the SV channel inhibition by Arachidonic Acid**

In order to gain a better understanding of the effects induced by Arachidonic Acid on the SV currents, we performed a dose-response characterization in the concentration range from 1 to 10 µM of AA in carrot root vacuoles. The results obtained in such experiments are summarized in **Fig. 3.25** where $G_{\text{norm}}$ is plotted as a function of AA concentrations. Panel A shows the dose-response inhibition for selected voltages from +60 to +120 mV. Note that only four voltages are represented for clarity reasons. For all the potentials under investigation a fit curve was performed, which allows to determine a series of Hill constants, $K_h$ and Hill coefficient, $n$. Panel B displays the $K_h$ values, plotted against the applied membrane voltages. The Hill coefficient ($n$) was plotted against the applied membrane voltages; as shown in panel C, $n>1$ at all the investigated voltages.

One can observe that the data illustrating AA modifications of the SV currents, in **Figs. 3.24 and 3.25**, qualitatively remind the results obtained on the addition of Naringenin, illustrated in **Figs. 3.8 and 3.9**. Only the Hill inhibition constant, $K_h$, is significantly smaller (more than two order of magnitude) in the case of AA with respect to Nar.

Clearly, much lower concentrations of AA (in the micromolar range) are sufficient to induce an inhibition of the current comparable to that observed at millimolar concentrations of Naringenin.
Fig. 3.25. Quantitative analysis of the SV current inhibition by Arachidonic Acid: $G_{\text{norm}}$ plotted as a function of Naringenin concentrations. A) Dose-response inhibition in the voltage range from +50 to +120 mV; note that only four voltages are represented for clarity reasons. For all the potentials under investigation a fit curve was performed, which allows to fit the data sets with a series of Hill coefficients and Hill functions. B) $K_h$ values derived from the Hill fit (shown in panel A) are plotted against the applied membrane voltages. C) The Hill coefficient ($n$) was plotted against the applied membrane voltages; note that $n>1$ at all the investigated voltages. Error bars indicate SEM.
Ch. 3.16. **Arachidonic Acid effects on the SV single channel conductance**

To investigate whether AA binds within the pore of the SV channel, we performed single channel experiments on vacuoles from carrot roots with 3 µM AA. The protocol was based on fast voltage ramps in order to get access to the conductance at far positive voltages where single channel cannot be resolved because of high number of open channels. In Fig 3.26 the single channel currents obtained from voltage ramps in the absence (black trace) and in the presence of 3 µM AA (red trace).

![Graph](image)

**Fig. 3.26.** Single channel current–voltage relationships obtained after the application of a fast voltage-ramp in control (black trace) superimposed to data (red trace) obtained in the presence of 3 µM AA in cytosolic side-out excised patch from the vacuoles of carrot roots. Solutions used were standard control solution 3 (see materials and methods).

These data demonstrate that the amplitude of the single channel conductance does not change in the presence or absence or AA.
Discussion
The majority of plant cells contains organelles called vacuoles surrounded by a membrane called tonoplast. In mature plant cells, the vacuole occupies up to 90% of the cell volume. The transport across the tonoplast is crucial for cell homeostasis and to maintain the turgor pressure which is fundamental in the regulation of cell expansion and growth (Maeshima 2001). The vacuole stores the essential compounds, minerals, nutrients, metabolites and different ions required for cell homeostasis. Different proteins of the tonoplast allow the ions to selectively permeate according to their electrochemical gradients (channels) or at the expense of other energy sources (active transporters).

**The Slow Vacuolar channel**

The patch-clamp technique offers the possibility of a direct examination of ion channel properties in isolated vacuoles. The SV channel was shown to be calcium activated in vacuoles from beet storage tissues (Hedrich and Neher 1987) and was subsequently characterized in vacuoles from many plant species (Allen and Sanders 1997; Carpaneto et al. 1997; Scholz-Starke et al. 2005a; Dziubinska et al. 2008). Nowadays it is generally accepted that the SV channel is ubiquitous in higher plants. Typical features of the SV channel are the outward rectification at elevated unphysiological cytoplasmic Ca\(^{2+}\) concentrations and the slow activation. Indeed it is well known that the SV currents recorded in a patch-clamp experiment require unphysiologically high cytosolic and low vacuolar calcium concentrations for full activation. The SV channel is a non-selective cation channel, permeable to both monovalent and divalent cations (Pantoja et al. 1992; Ward and Schroeder 1994; Allen and Sanders 1996; Gambale et al. 1996; Ivashikina and Hedrich 2005). It has been demonstrated that the SV channel, besides being permeable to potassium ions, is also permeable to calcium (Gradogna et al. 2009). It has also been demonstrated that the SV channel is encoded by the TPC1 gene (Peiter et al. 2005), comprising 12 alpha-helices and two pore segments.

It is well known that the SV channel is modulated by a series of different parameters and compounds, internal and external to the vacuole; beside calcium, Mg\(^{2+}\), protons, redox potentials and various metals like Zn\(^{2+}\) and Ni\(^{2+}\) and the glycosidic antibiotic Neomycin (Scholz-Starke et al. 2006) are able to modify the properties of the SV channel. However, as none of these parameters was able to transform the SV into a channel that activates at physiological potentials and calcium concentrations, we aim at investigating unexplored
working conditions or compounds, which are able to further modulate this channel. Specifically we plan to find out endogenous plant substances (for example flavonoids, glycosidic compounds, polyamines and other charged or uncharged molecules, reducing and oxidizing molecules, etc...) which might be able to modify the voltage activation threshold of this channel towards more physiological conditions or that provide information on the role and the nature of this enigmatic channel.

**Flavonoids and polyunsaturated fatty acids**
Flavonoids are plant pigments or secondary metabolites present in all plant species. They are one of the largest group of secondary metabolites represented by more than 8000 different compounds (Harborne and Williams 2000; Ververidis et al. 2007). It was reported that the flavanone Naringenin (Nar) is present in all plants where it plays a central role in the biosynthetic pathway (Lepiniec et al. 2006).

**Flavonoid localization**
It is generally believed that flavonoids are synthesized on the multienzyme complexes at the cytoplasmic surface of the ER from where they are transferred to different locations. However their glycosylation site is not well known so far. Therefore we firstly investigated the presence of non-glycosylated Nar in the cytoplasm and then studied the effects of Naringenin on the SV channel by the patch-clamp technique. In order to confirm that free Naringenin is present in the cytoplasm, UDP-glucosyl transferase gene, which glycosilates Naringenin into Naringin, was isolated. *Arabidopsis thaliana* UDP-glucuronosyl/UDP-glucosyl transferase family protein (AT1G06000) (AtGT) was amplified using genomic DNA as a template, cloned in frame with YFP as a fusion protein in the pGreen vector. The subcellular localization was investigated by PEG transformation of *Arabidopsis* mesophyll protoplasts with pGreen AtGT-YFP vector and by agroinfiltration of tobacco leaves. It was shown that the AtGT-YFP fusion protein is mainly expressed in the cytoplasm, thus confirming that non-glycosylated Naringenin is present in the cytoplasm.

**Modulation of SV channel by flavonoids**
We investigated the modulation of the SV channel by the flavonoid Nar in vacuoles from carrot roots and *Arabidopsis thaliana* mesophyll cells by using the patch-clamp technique. When Nar was added to the cytosolic bath solution in carrot root vacuoles we recorded a dose-dependent reversible decrease in SV channel activity described by the
Hill equation with \( n > 1 \) and a half block concentration of about 0.4 mM. Similar effects in the channel activity were observed in vacuoles from mesophyll cells of *Arabidopsis thaliana*.

Naringenin changed the characteristics of SV channel activation “unfortunately” shifting toward more positive membrane potentials the activation threshold and decreased the deactivation times. This implies a diverse modification of the voltage sensor during the opening and the closure processes.

In accordance with the macroscopic current measurements we also verified that Nar did not change the amplitude of the single channel conductance but modified the opening probability of the channel favoring its closed state. Indeed, the mean open time decreased and the mean closed time of the channel increased significantly in the presence of cytosolic Nar.

In order to verify whether Nar affects both potassium and calcium conductance, we performed experiments by combining the patch-clamp technique with fluorescence measurements using the fluorophore fura-2. When 200 µM Nar was added to the bath solution we could observe a reversible decrease in the current as well as in the fluorescence signals. In the presence of 1000 µM Nar the current and fluorescence signals were completely and reversibly abolished. Therefore Nar affects both K\(^+\) and Ca\(^{2+}\) permeation.

When we investigated the effects induced by 1000 µM Nar added at the luminal side we observed a reversible inhibition of the initial SV current of about 40% in vacuoles from both carrot roots and mesophyll cells of *Arabidopsis thaliana*.

We also performed experiments on SV currents from carrot root vacuoles with other flavonoids, like Apigenin and Chrysin, which share similar structural properties with Naringenin. We observed a reversible decrease in the activity of the channel when 300 µM Apigenin and Chrysin were added at the cytosolic side.

Finally, in order to verify the specificity of Nar inhibition, we performed experiments using Naringin, the glycosylated form of Naringenin, on vacuoles from *Arabidopsis thaliana* mesophyll cells. Interestingly Naringin did not induce any significant decrease of the channel activity even at a concentration of 1000 µM added both at the cytosolic side or the luminal side. In conclusion only the non-glycosylated form, Naringenin did affect the channel activity. We hypothesize that this difference depends on the higher
hydrophobicity of the nonglycosylated flavonoid with respect to the glycosylated form that carries a relatively large hydrophilic sugar terminal.

Indeed it has been reported that Nar can partition in the hydrophobic core of the lipid bilayer (van Dijk et al. 2000) modulating the membrane fluidity (Arora et al. 2000). It has also been demonstrated that flavonoids could penetrate into the lipid bilayer to different extents at different pHs. For example the flavonoid Quercetin (QCT) is deeply embedded in planar lipid bilayer intercalating the flexible acyl chains at acidic pH (Movileanu et al. 2000) where QCT is neutral and highly hydrophobic. Instead at more alkaline pHs, where QCT is deprotonated, the flavonoids binding site is restricted to the hydrophilic domain of the membrane (Terao et al. 1994; Movileanu et al. 2000). In order to further investigate these aspects we changed the pH of the external solution from 7.5 to 6.5. Under these conditions, we challenged the SV currents with 500 µM Nar. Interestingly at pH 6.5 the inhibition of SV the channel was larger compared to the inhibition at pH 7.5. All these indications suggest that Nar might interact with the SV channel through the phospholipid bilayer. More studies are needed to support this possibility.

**Modulation of the SV channel by PUFAs**

For this reason we investigated the interaction of the carrot root SV channel with polyunsaturated fatty acids (PUFAs). Arachidonic acid (AA, 5,8,11,14- all-cis-eicosatetraenoic acid) and Docosahexaenoic acid (DHA, 4,7,10,13,16,19-all-cis-docosahexaenoic acid) were added at the cytoplasmic side of the vacuole to investigate the response of the SV channel. Similarly to Naringenin micromolar concentrations of PUFAs induced a dose-dependent reversible decrease in SV channel activity.

In conclusion our data strongly suggests that the SV channel is modulated by flavonoids and by other compounds, such as PUFAs, that interact with the protein through the lipid bilayer. It is not clear whether these compounds modify the SV channel properties directly or changing the organization and fluidity of the bilayer.

Indeed a specific drug might modulate the channel properties either interacting with the protein at the water-membrane interface or, in alternative, binding either at a site internal to the protein or deeply embedded into the lipid bilayer. In general the modifications of channel properties may typically occur as a consequence of a direct binding and occlusion of the pore or by a modification of either the permeation pathway or the gating mechanisms. The first process is typically fast and is influenced by the nature of the
permeating ions present in the water solution when the channel is challenged with the drug. Instead the second is a slower process that depends on the physico-chemical characteristics of the protein as well as on the membrane structure. Indeed it is well known that several hydrophobic compounds, such as dihydropyridines, are able to block the L-type calcium channels via a lipid mediated interaction (Gambale and Dellacasagrande 1994). This block and the reversibility of the blocking mechanism mediated by different dihydropyridines, in some cases, may give rise to long lasting inhibition of the channel.

Several cases are reported where phospholipids, PUFAs, phosphoinositides, cholesterol and other endogenous compounds participate, alone or together with beta subunits, in the modulation of the transport characteristics of the channel.

For example it was reported (Roberts-Crowley and Rittenhouse 2009) that Arachidonic Acid modulates the properties of the L-type calcium channel CaV1.3b in superior cervical ganglion neuron in a manner that highly resembles the action of M1 muscarinic receptor. AA decreases the open probability of CaV1.3 (Liu and Rittenhouse 2000) and T type CaV1.3 (Talavera et al. 2004) channels, increasing the dwell time in the closed state without affecting the single channel conductance amplitude (Liu and Rittenhouse 2000). Notably, this behavior strongly reminds the action of Naringenin and AA on the voltage-dependent calcium permeable SV channel (see Materials and Methods, Chs. 3.6 and 3.13). It was also demonstrated that AA inhibits and changes the kinetics of CaV1.3 channel directly and not through metabolites or the intermediates of CaV β subunits. Interestingly palmitic acid may compete with AA for an inhibitory site, responsible for the AA-mediated current-decrease, located in the most hydrophobic part of the channel. This indicates that the membrane composition and AA may have competing or synergistic effects on the channel.

Another example of modulation of channel properties by intracellular AA is the inhibition of the voltage-dependent K+ channels Kv1.4 or Kv4.2 which is prevented by antioxidants (Angelova et al. 2009). This suggests the presence of diverse oxidative sites playing important roles in the properties of these K+ voltage-dependent channels. As it is well known that also Slow Vacuolar currents are sensitive to the redox potential, in future experiments the possibility that AA affects the SV channel properties through a
modification of redox reaction sites should be monitored. Likewise one should also investigate any potential interaction between flavonoids and antioxidant AA.

The polyunsaturated fatty acids AA and its amide, Anandamide, produce a rapid inactivation of K_V channels (Oliver et al. 2004). The relatively slow modification induced by AA and Anandamide strongly suggest that the interaction is mediated by the lipid membrane and prevented by Cs^+ and NH_4^+ present in the permeation pore.

CONCLUSIONS

Naringenin effects on the SV channel, namely the kinetics and the voltage dependence of macroscopic currents (Chs. 3.3 and 3.5) and single channel data (Ch. 3.6), the comparable inhibition of both Ca^{2+} and K^+ permeation (Ch. 3.7), the dependence of inhibition on the pH of the cytosolic solution (Ch. 3.8), the comparable activity when Naringenin is added either at the cytosolic or at the vacuolar side of the tonoplast (Ch. 3.9), the absence of inhibition by Naringin (Ch. 3.10) as well as PUFAs and Naringenin comparable effects point to a significant role played by the membrane composition, organization and fluidity in modulating the interaction of the SV channel by flavonoids and polyunsaturated fatty acids.

More experiments are needed to quantify the interaction between Naringenin and Arachidonic Acid with specific protein binding sites as well as the contribution of the phospholipid hydrophobic core and the role of the surface charges and/or the organization of the lipid polar heads at the membrane water interface. For example, the Arabidopsis mutant plant fou2 presents a single aminoacid substitution on the SV channel that shifts the threshold of channel activation towards negative voltages. fou2 has been shown to upregulate oxylipins in the jasmonate pathway where linolenic acid is released from the plasma membrane to be converted in jasmonic acid.

On the other side, PUFAs inhibit the SV channel with high affinity and in a voltage dependent manner. We can speculate that, if linolenic acid acts similarly to AA and DHA, its removal from the vacuolar membrane too might unlock the SV channel. In any case a mechanism of lipid mediated feedback involving the SV channel may occur.

These results are surprising and interesting as they open new perspectives on the role of the SV channel in vivo in different membranes with diverse phospholipid compositions. Therefore biophysical characterization opens new perspectives on the physiological properties of the SV channel in different plants and tissues.
References


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