Running head:
A link between flavonoid biosynthesis & transport

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**Flavonoid biosynthesis in barley (**Hordeum vulgare** L.) primary leaves requires the presence of the vacuole and controls the activity of vacuolar flavonoid transport.**

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Keywords

Barley mutant line ant310, naringenin feeding, secondary metabolism, flavonoid transport, transport regulation, metabolic control
Abstract

Barley (*Hordeum vulgare*) primary leaves synthesize saponarin, a twofold glucosylated flavone (apigenin 6-C-glucosyl-7-O-glucoside), which is efficiently accumulated in vacuoles via a transport mechanism driven by the proton gradient. Vacuoles isolated from mesophyll protoplasts of the plant line *ant310*, which contains a mutation in the chalcone isomerase (*CHI*) gene that largely inhibits flavonoid biosynthesis, exhibit strongly reduced transport activity for saponarin and its precursor isovitexin (apigenin 6-C-glucoside). Incubation of *ant310* primary leaf segments or isolated mesophyll protoplasts with naringenin, the product of the CHI reaction, restores saponarin biosynthesis almost completely, up to levels of the wildtype Ca33787. During reconstitution, saponarin accumulates to more than 90% in the vacuole. The capacity to synthesize saponarin from naringenin is strongly reduced in *ant310* miniprotoplasts containing no central vacuole. Leaf segments and protoplasts from *ant310* treated with naringenin, showed strong reactivation of saponarin or isovitexin uptake by vacuoles while the activity of the UDP-glucose: isovitexin 7-O-glucosyltransferase was not changed by this treatment. Our results demonstrate that efficient vacuolar flavonoid transport is linked to intact flavonoid biosynthesis in barley. Intact flavonoid biosynthesis exerts control over the activity of the vacuolar flavonoid/H⁺-antiporter. Thus, the barley *ant310* mutant represents a novel model system to study the interplay between flavonoid biosynthesis and the vacuolar storage mechanism.
Introduction

One of the major challenges of plant physiology in the post-genome era is to understand the overall regulation of metabolite flow in different pathways in response to developmental, tissue- or cell-specific programs or environmental factors such as light or stress (Sweetlove and Fernie, 2005). Metabolic pathways often span more than one compartment at the cellular level. Compartmentation and controlled transmembrane metabolite transport hold the potential to establish the optimal concentrations of substances necessary for further enzymatic transformation in individual compartments. Subcellular compartmentation of metabolites results in individual pools of different sizes and is important for the local maintenance of high metabolite concentrations or for the separation of competing pathways such as anabolic and catabolic processes (Winkel, 2004). Thus, the analysis of biochemical pathways must also take account of transport processes and the spatial/subcellular organization of enzymes that catalyze consecutive synthetic reactions (Jorgensen et al., 2005). Despite the importance of transport steps for biosynthetic processes or the detoxification of xenobiotics, the mechanisms that allow a metabolic pathway to directly or indirectly control transport activities are largely unknown. This is reflected by the notion that under normal physiological conditions, transport steps are not considered rate limiting.

Flavonoid biosynthesis gives rise to structurally related, but functionally diverse compounds such as flavonols, flavones, anthocyanins, proanthocyanidins or isoflavonoids (Dixon and Paiva, 1995; Winkel-Shirley, 2001; Dixon et al., 2005) and represents an ideal model to study metabolic regulation in plant cells. Flavonoid synthesis branches from the general phenylpropanoid pathway and is subject to multiple levels of regulation, including transcriptional (Weisshaar and Jenkins, 1998; Lepiniec et al., 2006) and enzymatic regulation. Chalcone synthase (CHS), for example, catalyzes the first committed step in flavonoid synthesis and is regulated both at the level of enzyme activity (Knogge et al., 1986) and transcription (Block et al., 1990; Hartmann et al., 1998; Hartmann et al., 2005). CHS also responds strongly to environmental stimuli such as light or elicitors (Chappell and Hahlbrock, 1984; Schulze-Lefert et al., 1989; Christie and Jenkins, 1996; Faktor et al., 1997). In fact, the whole pathway is regulated developmentally- (Schulz and Weissenböck, 1988), in specific tissues or cell-types (Schulz and Weissenböck, 1986; Hutzler et al., 1998) and is affected by stress (e.g. by UV; Reuber et al., 1996).

Upon synthesis, many conjugated products of the flavonoid pathway such as flavonol and flavone glycosides or anthocyanins are found predominantly in the
vacuole (Graham, 1998), while in other cases conjugated and/or unconjugated flavonoids are secreted into the apoplastic space (Onyilagha and Grotewold, 2004). Isoflavonoids, for example, are secreted by the roots of legumes where they influence the interaction of the plant with microbes (Paiva, 2000). Isoflavonoid secretion affects the surrounding microflora in the rhizosphere (Weisskopf et al., 2006), attracts mutualistic microorganisms such as N-fixing bacteria and enhances defense responses to pathogens (Paiva, 2000; Dakora and Phillips, 2002). In other cases, flavonol glycosides have been detected in the cell wall fraction of Scots pine needles (kaempferol 3-glucoside) (Strack et al., 1988) and Chrysosplenium americanum (polymethylated flavonol glucosides) (Ibrahim et al., 1987; Schnitzler et al., 1996) while flavonoid aglycones including flavones and flavonols have been detected in exudates of numerous species (Valant-Vetschera and Wollenweber, 2001; Wollenweber et al., 2003).

It is generally accepted that flavonoid biosynthesis largely takes place in the cytosol. The prevailing spatial model suggests that the early proteins of the pathway are organized in a multi-enzyme complex centered around cytP450-dependent monooxygenases on the cytosolic side of the endoplasmic reticulum (ER) forming a functional metabolon (reviewed by Saslowsky and Winkel-Shirley, 2001; Winkel, 2004; Jorgensen et al., 2005). Since histochemical analysis has repeatedly indicated that flavonoids are present in the nucleus (e.g. Peer et al., 2001; Saslowsky et al., 2005) and recent reports suggest also the presence of CHS and chalcone isomerase (CHI), the function of flavonoids as regulators in the nucleus suggest additional layers of complexity and compartmentation in the process (Saslowsky et al., 2005). Interestingly, aureusidin synthase from Antirrhinum majus flowers, a polyphenol oxidase catalyzing the formation of aurones from chalcones, has recently been localized to vacuoles (Ono et al., 2006).

In contrast to our knowledge on flavonoid biosynthetic enzymes and the transcriptional regulation of the corresponding genes, the transport of phenolic compounds into subcellular compartments, facilitating the protection of cytosolic processes against the intrinsic toxicity of these compounds, is only marginally understood both at the mechanistic and regulation levels. While transport of flavonoids across the plasma membrane has not been investigated so far, biochemical analysis of vacuolar uptake mechanisms led to the proposition of different transport and accumulation mechanisms (reviewed by Grotewold, 2004; Yazaki, 2005). Available data suggest that the conjugated moieties such as sugars or acyl residues are important
determinants of transport specificity (Matern et al., 1986; Hopp and Seitz, 1987; Klein et al., 1996).

In barley the 2-fold glucosylated saponarin (apigenin 6-C-glucosyl-7-O-glucoside) accumulates as the major compound during primary leaf development (Figure 1; Reuber et al., 1996). Saponarin is synthesized from its precursor isovitexin (apigenin 6-C-glucoside) after the addition of glucose (glc) in the 7-O position catalyzed by a soluble UDP-glc:flavone glucosyltransferase (Blume et al., 1979). Compared with saponarin, isovitexin is present only in trace amounts. Both, saponarin and isovitexin are transported into and stored in barley vacuoles by a secondary energized proton antiport mechanism (Klein et al., 1996; Frangne et al., 2002).

Arabidopsis synthesizes conjugated flavonols instead of flavones (Graham, 1998). In contrast to the vacuolar saponarin/H+-antiport found when barley vacuoles are used, uptake of saponarin into Arabidopsis cell culture vacuoles is ATP-dependent and occurs via direct energization. The same has been found for the uptake of an abiotic glucoside, hydroxyprimisulfuron glucoside, which is synthesized during the detoxification of the sulfonylurea-type herbicide primisulfuron. Thus, an ATP-binding cassette (ABC) protein-type transporter driving vacuolar uptake has to be hypothesized in addition to a proton antiport mechanism in certain cases (Klein et al., 1996; Frangne et al., 2002). On the molecular level, as well ABC-type transporters as secondary energized multidrug and toxic exchange transporters (MATE) have been proposed to be involved in the transport of anthocyanins in maize or proanthocyanidins in Arabidopsis, respectively (Debeaujon et al., 2001; Dean et al., 2005).

A model system that allows the analysis of a link between a metabolic pathway and the transport processes involved in metabolite translocation into compartments or organelles should fulfill several preconditions: (i) It must be possible to control and manipulate the flow through the pathway experimentally. In other words, it must be possible to ‘turn’ the pathway ‘on’ and ‘off’. This can be done by selecting a mutant plant line with a lesion in a gene coding an enzyme involved in an early step in the pathway. This would allow the pathway to be switched on by applying the chemical product of the affected enzymatic reaction exogenously to effectively complement the mutation. (ii) If the transport activity investigated is controlled by the flow through the metabolic pathway, transport is expected to decrease or increase in response to the absence (mutant) or presence of metabolites (chemically complemented mutant. (iii) Organelle or membrane isolation must be facile and quick in order to study transport
activity changes and storage efficiency in response to alterations in the biosynthetic pathway.

Here we present an experimental model to investigate the metabolic control mechanism of the flavonoid biosynthetic pathway through the vacuolar transport system. We use the barley ant310 mutant, which accumulates less than 5% of the flavonoids of the wildtype Ca33787 (Reuber et al., 1996). Instead, mutant primary leaves contain isovalipunipose, which is a glucosylated form of 4, 2’, 4’ 6’-tetrahydroxyxalcone lacking the ring closure in the heterocyclus to flavanones, a step catalyzed by CHI (Figure 1; Reuber et al., 1997). By diallelic crosses, four independent mutants including ant310 were found to belong to the same complementation group (Reuber et al., 1997). Using the maize CHI gene as a probe, Druka et al., (2003) identified a barley ortholog and showed that the ant310 mutant lacked a functional CHI. Surprisingly, transport of isovitexin and saponarin into vacuoles isolated from ant310 primary leaves was strongly reduced when compared to vacuoles isolated from Ca33787 (Frangne et al., 2002). This result indicated that the intact flavonoid pathway was necessary for fully-activated vacuolar flavonoid/H⁺-antiport activity. Here we show that micromolar concentrations of exogenously added naringenin, the product of the CHI reaction, restores saponarin biosynthesis in ant310 leaves to levels of the wildtype in only 5-6 hours. Reconstitution of saponarin production is cell-type autonomous since isolated ant310 mesophyll protoplasts are able to synthesize saponarin after naringenin addition. Naringenin-induced saponarin biosynthesis in ant310 protoplasts depends on the presence of the vacuole as a ‘destination’ compartment and is strongly compromised if pH-gradients necessary for vacuolar H⁺-antiport transport activities are disrupted. Most importantly, naringenin-reconstitution of saponarin biosynthesis in ant310 reactivates the vacuolar transport activity for the barley flavone glucosides. We propose that the high-activity state of the vacuolar transporter is maintained by the biosynthesis of flavonoids in the pathway while the presence of a mature lytic vacuole controls the metabolite flow.
Results

Feeding of ant310 leaf segments with naringenin fully reconstitutes saponarin biosynthesis.

When naringenin was fed externally to segments of 4 d old ant310 primary leaves, efficient saponarin biosynthesis was observed within 7 h of incubation (Figure 2). In the presence of 50 µM naringenin in the medium, full reconstitution of saponarin biosynthesis was observed since saponarin levels reached the amount present in the wild-type Ca33787 leaf segments incubated in the absence of naringenin. Saponarin production was absent in ant310 leaves on control medium indicating that presence of naringenin was necessary for saponarin production. Furthermore, feeding of ant310 leaves with 100 µM naringenin resulted in less efficient saponarin production which suggested that 50 µM naringenin were sufficient for efficient saponarin synthesis while higher concentrations could be toxic. Saponarin production from naringenin in ant310 leaves reached similar levels in 5 and 6 d old primary leaf segments while the biosynthesis was reduced in 7 or 8 d old leaves. In the latter leaves, about 30 % of saponarin was formed from naringenin when compared to the corresponding Ca33787 flavonoid content (data not shown). In contrast, secretion of saponarin from leaf segments into the incubation medium never exceeded 5 % of the amount formed within the tissue, suggesting that the flavonoids produced in ant310 leaves in the presence of naringenin were efficiently stored within the cells. This result prompted us to investigate the cell-autonomy of saponarin reconstitution and vacuolar compartmentation.

For all experiments with protoplast and fractions derived thereof, 7-8 d old primary leaves were used. Older primary leaves were chosen (a) because protoplast and vacuole experiments required the preparation of large amounts of primary leaf material and (b) because large-scale isolation of protoplasts and vacuoles from younger leaves is difficult. Starch production is marginal in young leaves, which results in the loss of mesophyll protoplasts during density centrifugation and fraction purification. In any case saponarin production was still accurately measurable by HPLC in protoplasts derived from 7-8 d old leaves.

Saponarin biosynthesis from naringenin in ant310 is cell-autonomous.

Mesophyll protoplasts isolated from 7 d old ant310 primary leaves were incubated in the absence or presence of 50 µM naringenin and the content of saponarin
in protoplasts was analyzed by HPLC in a time-course study. As for the leaf segments, ant310 protoplasts were able to synthesize saponarin in the presence of naringenin reaching maximal levels after 2-3 h of incubation while ant310 protoplasts kept without naringenin did not synthesize saponarin (Figure 3). As observed for leaf segments, protoplasts isolated from 7 d old ant310 leaves reached about 25-30 % of the saponarin content of Ca33787 protoplasts (not shown). We investigated whether the saponarin synthesized in ant310 protoplasts during naringenin feeding was stored within the vacuole by performing a compartmentation analysis. The saponarin content was measured by HPLC in protoplast and vacuolar fractions isolated after 6 h of feeding ant310 protoplasts with naringenin. In order to compare protoplasts and vacuoles, we determined acid phosphatase activity as a vacuolar marker enzyme in the same samples. Reconstituted saponarin synthesized during naringenin incubation of ant310 protoplasts was efficiently transferred into the vacuole because 95±3 % of the saponarin formed during naringenin feeding was found in the vacuolar fraction. Thus, in contrast to our former saponarin transport experiments into ant310 vacuoles (Frangne et al., 2002), the tonoplast transport system for saponarin appeared to be fully active in ant310 protoplasts in the presence of naringenin-driven saponarin biosynthesis.

We used diphenylboric acid-2-aminoethyl ester (DPBA) to microscopically visualize saponarin accumulation in ant310 protoplasts during reconstitution from naringenin, but found that the fluorescence signal obtained does not appropriately detect the vacuolar localization of the barley flavonoids in mesophyll protoplasts (data not shown).

**Saponarin biosynthesis from naringenin requires the presence of the central vacuole and is independent from de-novo protein biosynthesis.**

Saponarin was formed during naringenin-feeding of ant310 protoplasts and was efficiently stored in the vacuole. We, therefore, examined whether presence of the vacuole was a prerequisite for saponarin biosynthesis from naringenin. We prepared evacuolated miniprotoplasts from ant310 mesophyll protoplasts lacking the central, acidic vacuole as seen by the absence of a neutral red stained compartment in miniprotoplasts (data not shown; (Hörtensteiner et al., 1992). The efficiency with which central lytic vacuole was removed during evacuolisation was further assessed by measuring the activities of different vacuolar hydrolytic enzymes in protoplast and miniprotoplast fractions. When compared to protoplasts (100 %), evacuolated miniprotoplasts contained only 3.1±0.6, 0.3±0.2 and 1.2±1.0 % of the activities of acid...
phosphatase, β-N-acetylglucosaminidase and α-mannosidase, respectively. In contrast to some dicotyledonous plants, barley was not able to regenerate a new vacuole. When evacuolated miniprotoplasts isolated from ant310 leaves were incubated with naringenin, only very low amounts of saponarin were detected within 6 h, while the corresponding protoplasts reached maximal saponarin levels again after 2 h (Figure 4). We concluded that the presence of the vacuole as a destination compartment for saponarin accumulation was necessary for saponarin accumulation.

Since saponarin biosynthesis from naringenin in ant310 was a rather quick process, already observed within up to 30 minutes (Figures 3, 4), we reasoned that the absence of CHI in the mutant did not affect the amount or in situ activity of the biosynthetic enzymes downstream of the isomerase. Indeed, addition of cycloheximid to ant310 protoplasts did not affect saponarin production from externally fed naringenin, suggesting that de-novo protein biosynthesis was not required for saponarin biosynthesis and vacuolar storage (Figure 5). In contrast, when CCCP or NH₄Cl, which dissipate pH gradients across membranes, were added to ant310 protoplasts in the presence of naringenin, saponarin production was reduced by about 50% to values observed with protoplasts incubated on ice during naringenin treatment. Changes in the electrical membrane potential, caused by the addition of the K⁺-ionophore valinomycin, did not affect saponarin biosynthesis in naringenin-fed ant310 protoplasts (Figure 5). Thus, the overall inhibitor sensitivity of saponarin reconstitution in ant310 protoplasts resembled the pharmacological profile of the vacuolar saponarin/H⁺-antiporter (Frangne et al., 2002). Based on the absence of saponarin production in miniprotoplasts together with our previous observation of strongly decreased saponarin transport activity in isolated ant310 vacuoles, we hypothesized that the absence of the vacuole or a decrease in the activity of vacuolar transport reduces the capacity to synthesize saponarin.

Naringenin-feeding of ant310 reactivates the vacuolar flavone glucoside transporter.

Saponarin was efficiently stored in the vacuole during incubation of ant310 protoplasts with naringenin. Thus, the vacuolar transport system should not have limited the overall biosynthetic capacity. In view of our previous data demonstrating strongly reduced saponarin transport in ant310 vacuoles (Frangne et al., 2002), we tested whether resuming flavonoid biosynthesis in ant310, affected the activity of the vacuolar flavone glucoside/H⁺ antiporter.
We incubated ant310 leaf segments and protoplasts without or with 50 µM naringenin for 3 h and isolated vacuoles in parallel followed by transport experiments. From our earlier experiments it was known that the precursor isovitexin as well as saponarin itself were transported into vacuoles of the barley wildtype by an H\(^+\)-antiport with \(K_m\) values of about 100 µM. Furthermore, saponarin and isovitexin competitively inhibited each others transport suggesting that both substances were taken up in barley vacuoles by the same transporter (Klein et al., 1996; Frangne et al., 2002). We, therefore, investigated uptake of both substances, isovitexin and saponarin, into ant310 vacuoles after feeding the protoplasts with naringenin (Klein et al., 1996) and substrate concentrations both at the \(K_m\) value (100 µM).

In accordance with our former observation (Frangne et al., 2002), isovitexin was not taken up by vacuoles isolated from untreated ant310 leaf segments. Figure 6A displays an HPLC trace of vacuoles after 18 min incubation with isovitexin in the presence of MgATP, but without prior naringenin-feeding of leaf segments. The only remarkable peak (denoted as peak no. 2) corresponded to the ant310-specific compound isosalipurposid. In contrast, vacuoles isolated from naringenin-treated ant310 material (3 h) exhibited after 18 min of uptake and presence of MgATP a peak (no. 1 in Figure 6) that corresponded to authentic isovitexin. Figure 6 C displays a comparable, time-dependent experiment with saponarin as substrate for the vacuolar uptake experiments. Only vacuoles isolated from ant310 leaf segments, preincubated with naringenin, exhibited the vacuolar saponarin transport activity. The overall velocities of isovitexin and saponarin transport into vacuoles of naringenin-treated ant310 leaves were comparable to expected transport rates for Ca33787 vacuoles calculated from our published kinetic constants for 100 µM substrate (Figure 6 C, D; see (Frangne et al., 2002). Furthermore, reactivation of vacuolar saponarin transport was independent of the time-point of naringenin incubation during the isolation procedure: Results were identical when naringenin was added either for 2-3 h during leaf segment preparation, cell wall digestion and protoplast isolation or immediately to the purified protoplasts.

The latter notion prompted us to investigate whether naringenin-feeding-induced vacuolar transport-reactivation in ant310, was a quick or slow process. For this experiment, ant310 protoplasts were isolated and incubated in parallel for 10 min and 2 h with naringenin, before vacuoles were isolated and saponarin transport activities were determined. As a control, vacuoles were prepared from ant310 protoplasts omitting naringenin treatment (2 h). As can be seen from Figure 7, 10 min of naringenin
treatment of protoplasts were not sufficient for an activation of the vacuolar saponarin transport activity when compared to vacuoles isolated after 2 h of protoplast feeding with naringenin. This result indicated that a metabolite derived from or induced by naringenin reactivated the vacuolar transport activity and not naringenin itself. We concluded, that resumed flavone glucoside biosynthesis in ant310 leaves by naringenin-feeding restored the vacuolar transporter for these flavone glucosides to a high degree.

Naringenin-feeding does not affect the activity of the UDP-glucose: isovitexin 7-O-glucosyltransferase.

Finally, we examined whether a metabolic regulation of a step in flavone glucoside biosynthesis was specific for the vacuolar transport system or also extended to other enzymes. As the only unequivocally defined step in late flavone glucoside biosynthesis in barley, we measured the activity of the UDP-glucose: isovitexin 7-O-glucosyltransferase (OGT) converting isovitexin to saponarin (Figure 1; (Blume et al., 1979). The activity of the OGT analyzed in crude extracts prepared from 5 d old leaf segments, was not affected either by the mutation in ant310 nor by incubation of ant310 leaf segments with naringenin (Table I). Thus, naringenin feeding of ant310 leaves did not have an effect on the activity of a soluble biosynthetic enzyme when compared to the vacuolar flavone glucoside transport system.
Discussion

We have previously shown that the vacuolar transport activity for the major barley flavone glucoside saponarin was strongly reduced when vacuoles isolated from ant310 leaves were compared to the corresponding wildtype (Frangne et al., 2002). The discovery that the mutation affected the CHI gene (Reuber et al., 1997; Druka et al., 2003) suggested that the absence of CHI activity in ant310 in some way inhibited the vacuolar flavonoid transport activity. In order to examine whether intermediate(s) or products of flavonoid biosynthesis stimulate vacuolar flavonoid transport, we first analyzed whether it is possible to chemically complement the chi mutation in ant310 by external application of naringenin.

We demonstrate that full chemical reconstitution of the flavonoid pathway by naringenin is possible in the ant310 mutant, that naringenin conversion to saponarin needs the presence of an intact vacuole and that reconstitution of the flavonoid pathway by naringenin in the ant310 mutant reactivates the vacuolar flavonoid transporter to a high degree. Thus, we present an experimental model system that allows the detailed analysis of a metabolic linkage between a biosynthetic pathway whose activity can be externally manipulated and a transport step that appears to be necessary for the full activity of the pathway.

Efficient chemical reconstitution of a chi mutant: Implications for the flavonoid biosynthesis metabolon.

The flavonoid biosynthetic pathway represents an important branch of the general phenylpropanoid pathway which also gives rise to other structurally and functionally diverse phenolic compounds including soluble and cell wall-bound hydroxycinnamic acids, monolignols/lignin, sinapate esters, coumarins, and simple phenolics such as benzoic acid and salicylic acid (Dixon and Paiva, 1995). All these compounds are ultimately derived from trans-cinnamic acid, which is produced via PAL. Since single cells are able to synthesize different products of the phenylpropanoid pathway at the same time, enzymes catalyzing the reactions at the branchpoints will compete for the precursors. Consequently, precursors should either be bound and converted with high affinity, exist in spatially separated cellular pools or be directly handed over to downstream enzymes most probably due to the close proximity of enzymes. In an attempt to explain competition for common intermediates, (Stafford, 1974) proposed that the different branches of phenylpropanoid metabolism are...
organized in enzyme complexes allowing channelled synthesis of different end-products (Winkel, 2004). To date, it has become apparent, using metabolite feeding in combination with transgenic approaches, (co-)localization and interaction assays that PAL and the proximate enzyme, cinnamate 4-hydroxylase (C4H) converting trans-cinnamic acid to 4-hydroxycinnamate are in close association on the ER facilitating metabolic channeling at the entry point of the phenylpropanoid pathway (Rasmussen and Dixon, 1999; Liu and Dixon, 2001; Achnine et al., 2004). Furthermore, in the flavonoid pathway, Arabidopsis CHS, CHI, flavonol 3-hydroxylase interact in association with the ER (Burbulis and Winkel-Shirley, 1999; Saslowsky and Winkel-Shirley, 2001). Channeling, gel filtration and cell fractionation studies performed by Hrazdina and Wagner (1985) suggested that a functionally intact multienzyme complex occurred loosely attached to the ER membrane, which contains all enzymes from PAL up to the late UDP-glc: flavonoid glucosyltransferase. As a consequence, the question arises at which biosynthetic step is efficient feeding of precursors or intermediates possible or, in other words, where and how many ‘entry points’ for metabolites exist along the channel.

Here, we demonstrate that exogenous addition of naringenin resulted in efficient synthesis of saponarin, the major flavone glucoside in barley ant310 primary leaves lacking functional CHI (Figure 2, 3). In the case of 4 d old primary leaves, 50 µM naringenin in the medium led to the production of levels of saponarin within 7 h of incubation that are present in the wildtype. This result suggests that naringenin is very efficiently taken up by barley leaf cells, is readily converted by all catalytic activities acting downstream of CHI and is not, therefore, limiting the rate of metabolite flow in the ant310 mutant. Furthermore, flavonoid production occurred in a cell-autonomous manner since saponarin was synthesized from naringenin in isolated ant310 mesophyll protoplasts (Figure 3, 4). Finally, the major end product is efficiently transferred into the vacuole while in evacuolated miniprotoplasts lacking the vacuole saponarin production from naringenin was completely blocked (Figure 4).

Studies where exogenous feeding of naringenin is used to analyse the functionality of the flavonoid pathway in intact plants are rare. Using illuminated buckwheat hypocotyls, Amrhein (1979) demonstrated that suppression of anthocyanin biosynthesis by the PAL inhibitor aminoxyacetate could be restored by exogenous addition of naringenin. Concentration-dependent experiments showed that 0.5 to 1 mM naringenin were needed to reverse the anthocyanin content to 70 % of the control while higher concentrations of naringenin were inhibitory. In our experiments, raising the
exogenous naringenin concentration from 50 to 100 µM resulted in less efficient saponarin synthesis. Thus, higher naringenin concentrations have to be considered as toxic to the biosynthetic machinery. In Arabidopsis, different phenotypic features have been restored by chemical complementation of the *transparent testa (tt)*4 mutant, which affects CHS and therefore lacks all flavonoids (Shirley et al., 1995), with submicromolar concentrations of naringenin. These include anthocyanin accumulation in seedling cotyledons (Shirley et al., 1995), DPBA-induced yellow/-green fluorescence indicating presence of flavonols and processes related to flavonoid-regulation of auxin transport (Murphy et al., 2000; Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004) Thus, with regard to the metabolon organization of the phenylpropanoid pathway, the question arises whether naringenin represents a substrate that can efficiently enter the ‘flavonoid biosynthesis channel’ downstream of phenylalanine or whether lack of CHS (*tt4*) or CHI (*ant310*) leads to a structural rearrangement of the entire multienzyme complex, opening the complex to an exogenous pool of substrates that cannot enter the channel if the complex contains all functional subunits in the wild-type situation. However, submicromolar concentrations of naringenin significantly decreased auxin transport in both, wild-type and *tt4* Arabidopsis seedlings, when compared to controls not treated with naringenin (Peer et al., 2004). This suggests that also in the wildtype low exogenously added concentrations of naringenin can still efficiently enter the metabolon and if converted to flavonols exert their regulatory role on auxin transport.

**Metabolic regulation of vacuolar flavonoid transport: How and why?**

A clear advantage of the barley *ant310* primary leaf experimental system to study linkage between flavonoid biosynthesis and vacuolar transport of its products, is the relative ease and speed of organelle isolation following the supply of the metabolic precursor. Although Arabidopsis or Petunia as genetically well-defined systems with regard to flavonoid biosynthesis offer more mutants in important biosynthetic genes, exogenous feeding of precursors followed by the isolation of lytic vacuoles in sufficient amounts for transport experiments, is presently only well established in barley. Furthermore, the primary leaf is a well-defined system with respect to flavonoid biosynthesis (Klein et al., 1996; Reuber et al., 1996; Frangne et al., 2002) although important conversion steps still need unequivocal biochemical elucidation (see Figure 1). Finally, mesophyll protoplasts and lytic vacuoles represent a homogenous cell or organelle type, an advantage over the use of Arabidopsis rosette leaves to produce protoplasts: the differences in flavonoid composition of spongy and palisade...
parenchyma has not been experimentally determined in this model plant nor are we aware of detailed investigations of flavonoid patterns in rosette leaves representing different developmental stages.

Here we provide experimental evidence that the vacuolar flavonoid transport system in barley is linked to functional flavonoid biosynthesis. In vacuoles isolated from ant310 leaves, uptake of saponarin and its precursor isovitexin was strongly reduced (Frangne et al., 2002). Uptake of these two flavone glucosides in barley vacuoles was previously characterized, occurring via a proton-antiport mechanism (Klein et al., 1996; Frangne et al., 2002). Furthermore, competitive inhibition of saponarin transport by isovitexin and vice versa as well as comparable $K_m$ values suggested that both flavone glucosides were transported into barley mesophyll vacuoles by a membrane protein accepting both compounds as substrates. In contrast, when ant310 leaf sections or protoplasts were supplied with naringenin prior to vacuole isolation, transport of saponarin and isovitexin was strongly reactivated from almost undetectable transport activity in the absence of naringenin. Taking all independent transport experiments into account, the vacuolar transport activities after naringenin incubation of ant310 were $18 \pm 6$ and $21 \pm 5$ µmol substrate / (min * l vacuolar volume)$^{-1}$ for isovitexin and saponarin, respectively. This suggests no preference of the transporter for saponarin or isovitexin at 100 µM substrate concentration (a value close to the $K_m$ values). Using our kinetic values published for $[^3]$H-saponarin transport into vacuoles isolated from var. Bakara (Frangne et al., 2002), we calculated that the expected vacuolar transport rate for the flavone glucoside concentration applied in the transport experiments here (100 µM) was 13 µmol saponarin / (min * l vacuolar volume)$^{-1}$. Thus, the addition of naringenin fully reconstituted the flavone glucoside transport activity in ant310 cells. We propose, therefore, that the vacuolar flavonoid transporter in barley is under the strict control of the functional flavonoid pathway. Since reconstitution of saponarin production, which was intimately linked to vacuolar compartmentation, was not affected by high concentrations of cycloheximide (Figure 5), the regulation should occur at posttranslational level. It is tempting to speculate that transport regulation involves an unknown pathway intermediate binding to the flavonoid transporter. Alternatively, it could be hypothesized that the vacuolar transporter itself represents a part of the flavonoid metabolon. Based on the fact that the OGT activity representing the last biosynthetic step is neither influenced by the loss of CHI activity nor by naringenin feeding of ant310 leaves (Table I), it can be argued that
regulation of the pathway by its intermediates may be rather specific for the transport step.

If the flavonoid pathway generates a metabolite regulating the transport step, it cannot be naringenin itself, because reactivation required a naringenin incubation period of several hours while short (10 min) exposure of ant310 protoplasts to naringenin was not sufficient for efficient transport reactivation (Figure 7). Furthermore, this metabolite must be absent in ant310 plants and should be specific for the flavonoid pathway. Transport reactivation occurred after naringenin-feeding of ant310 leaves and therefore in the presence of the mutant-specific compound isosalipurposide accumulating as a consequence of the absence of CHI. Consequently, isosalipurposide does not likely act as an inhibitor of vacuolar saponarin transport in ant310 leaves (Figures 1, 6).

For an alternative proposition, a physical integration of the transport step into the hypothesized metabolon, an intriguing problem has to be addressed: How can an ER-associated biosynthetic complex interact with a vacuolar transporter? In this respect it is interesting to note that cellular inclusions containing flavonoids that may move between different compartments have been repeatedly described in different plant species, e.g. in pathogen-challenged Sorghum bicolor (Snyder and Nicholson, 1990; Nielsen et al., 2004). Maize Black Mexican Sweet cells accumulate green and yellow auto-fluorescent bodies targeted to the cell wall and the vacuole, respectively, following expression of the Myb-type transcription factor P1 which continues along with production of C-glycosylflavones (Lin et al., 2003). In different species, anthocyanic vacuolar inclusions have been shown to be responsible for local high accumulation of anthocyanins, which is associated with color intensification (Pecket and Small, 1980; Markham et al., 2000; Irani and Grotewold, 2005). Furthermore, mutations in the genes TDS4 and AHA10 encoding leucoanthocyanidin dioxygenase and a P-type H+-ATPase in Arabidopsis thaliana, respectively, resulting in defects in proanthocyanidin biosynthesis in the seed coat, are associated with alterations in vacuolar morphology visualized after addition of a fluorescent dye normally labelling the central vacuole (Abrahams et al., 2003; Baxter et al., 2005). Likewise, overexpression of the R and C1 regulators of anthocyanin accumulation in maize cells induces alterations in the subcellular distribution and vacuolar organization of anthocyanins in a light-dependent manner, which is also associated with changes in vacuolar morphology (Irani and Grotewold, 2005). The question of how glutathione S-transferases, such as BZ2 in maize (Marrs et al., 1995), TT19 in Arabidopsis (Kitamura et al., 2004) or AN9 in...
petunia (Alfenito et al., 1998) contribute to the accumulation of flavonoids in the vacuole, remains unanswered. Despite overwhelming genetic evidence for the importance of these usually soluble enzymes in a late step of anthocyanin biosynthesis prior to vacuolar storage, their direct enzymatic action on flavonoids has not been established. Instead, a role as soluble flavonoid-binding proteins that could guide flavonoids in a protected manner through the cytosol towards the vacuole has been proposed (Mueller et al., 2000).

We did not observe any changes in vacuolar morphology in ant310 leaves or protoplasts, and also isolation of vacuoles was possible from flavonoid-free protoplasts. It must be assumed, therefore, that the morphology of the central vacuole responsible for saponarin storage is not altered in ant310. Nevertheless, it is possible that the molecularly unidentified flavonoid transporter could travel between two compartments: the ER where it could be activated by a component of the metabolon and where it also could accept the flavonoid substrate as a cargo and the vacuole where transport would occur. Alternatively, flavonoids produced at the ER could be accepted by soluble GSTs and handed over to the vacuolar transporter after traveling through the cytosol. Clearly, experiments testing these hypotheses will need to address the cellular localization of the transporter in response to naringenin treatment and the availability of substrates.

The absence and reduction of naringenin-induced saponarin biosynthesis in evacuolated ant310 miniprotoplasts (Figure 4) and NH₄Cl- or CCCP-treated ant310 protoplasts (Figure 5) suggest that an intact and acidic vacuole is required for saponarin biosynthesis. These experiments do not help determine which hypothesis, described above, is true, however, they do suggest that a ‘destination compartment’ is necessary for efficient flavonoid production. The efficiency of the elimination of products of the flavonoid pathway from the cytosol to the vacuole appears to affect overall biosynthesis, which presumably involves feedback inhibition. Feedback inhibition of flavonoid biosynthesis resulting from the failure to store flavonoid end-products in the vacuole, agrees with observations made in antisense experiments of the anthocyanin transporter ZmMRP3 or using the Arabidopsis tt12 mutation, which causes a lesion in the gene encoding a presumptive transporter for proanthocyanidin precursors (Debeaujon et al., 2001; Goodman et al., 2004). Together with the inducible vacuolar flavonoid transport system demonstrated here this indicates that flavonoid biosynthesis is functionally linked to the transport step and to vacuolar compartmentation of flavonoids.
Materials and Methods

Chemicals.

Naringenin was obtained from Sigma (Buchs, Switzerland) and dissolved in 40 % (v/v) EtOH/H₂O (stock 1 mg ml⁻¹). The glucosylated apigenins, saponarin and isovitexin, were obtained from Extrasynthese (Genay, France) and flavonoid as well as inhibitor stocks were dissolved in DMSO. Stock concentrations for saponarin and isovitexin were calculated after measuring the absorbance at 340 nm using the molar extinction coefficient for apigenin (ε<sub>apigenin, 340nm</sub> = 20'800 M⁻¹; (Weast, 1982). For the naringenin reconstitution experiments, controls without naringenin contained the corresponding amount of EtOH. All other flavonoid standards were taken from our laboratory collection. Cell-wall digesting enzymes were from Seishin Pharmaceuticals (Japan) and UDP-glucose from Boehringer (Mannheim, Germany). For chemicals used during protoplast or vacuole isolation and HPLC analysis, see (Klein et al., 1996; Frangne et al., 2002).

Plant material and growth condition.

The barley lines Ca33787 (wildtype) and the ant310 mutant (Jende-Strid, 1993; Reuber et al., 1997; Druka et al., 2003) were grown in standard soil (Einheitserde Typ ED 73) in growth cabinets. For the naringenin reconstitution experiments with entire leaf segments performed in Cologne, plants were grown with 13 h of fluorescent light (80 µE m⁻² s⁻¹) while plants used for protoplast, miniprotoplast and vacuole isolations performed in Zurich were grown with a photoperiod of 14 h. In both locations, phytotrons were set to 20°C and a relative humidity of 60-70 %.

Naringenin feeding of leaf segments and flavonoid extraction.

In order to achieve maximal turgescence, plants were well-watered ~30 min before primary leaf segments were cut once above the coleoptile and 1 cm from the tip. This resulted in leaf segments of 3-4 and 5-6 cm for 4 or 5 d old primary leaves, respectively. The abaxial epidermis was removed mechanically and leaf segments (4-8 segments per experiment depending on leaf age) were transferred with their abaxial side on 5 or 10 ml 0.1 M KPi buffer (pH7) in Ø 5 or 9 cm petri dishes, respectively. Reconstitution started by the addition of naringenin solution or solvent to the medium followed by incubation at room temperature on a shaker (50 rpm) close to a window with daylight for the times indicated. Following incubation, the segments were quickly
dried with filter paper and the sample fresh weight was determined. For flavonoid extraction, segment samples were pulverized in the presence of liquid N\textsubscript{2} and 5 ml per g FW of 50 % (v/v) MeOH/H\textsubscript{2}O were added followed by 15 min extraction at room temperature with occasional vortexing. Prior to HPLC analysis, extracts were centrifuged in 1.5 ml reaction tubes at 18'000 x g for 10 min. Investigation of flavonoid exsudates following during leaf segment incubation was performed by directly subjecting incubation media aliquots to HPLC analysis.

**Isolation and naringenin feeding of mesophyll protoplasts, vacuoles and evacuolated miniprotoplasts.**

In comparison to naringenin feeding of 4 or 5 d old leaf segments, 7 d old primary leaves were used for all protoplast experiments. Isolation of barley protoplasts and vacuoles followed published procedures (Rentsch and Martinoia, 1991) with the modifications reported by (Frangne et al., 2002). The purity of barley mesophyll vacuoles was recently reassessed for vacuoles used in proteomic experiments (Endler et al., 2006). Marker enzyme activities detected less than 1 % of chloroplast, mitochondrial or cytosolic contamination in the vacuolar compared to the protoplast fraction while Western-blot analysis with compartment-specific antibodies suggests absence of contaminating membranes and compartments from vacuolar preparations. Evacuolated miniprotoplasts isolated from barley mesophyll protoplasts were obtained as described for tobacco (Hörtensteiner et al., 1992).

When protoplasts were used for naringenin feeding without subsequent vacuole isolation, protoplasts were purified using the Percoll step gradient described by (Rentsch and Martinoia, 1991) but 0.5M sorbitol / 1 mM CaCl\textsubscript{2} / 10 mM MES/KOH pH 5.6 (medium A) was used instead of 0.4M sorbitol / 30mM KCl / 5 mM HEPES/KOH pH 7.2 which was prepared for the isolation of vacuoles from protoplasts.

Naringenin feeding experiments with Ca33787 and ant310 protoplasts and miniprotoplasts were performed by adding naringenin to a final concentration of 50 µM to the illuminated cells kept in medium A. At times indicated, triplicate 0.25 ml aliquots were removed and added to 1.5 ml 0.5M glycine betaine / 1 mM CaCl\textsubscript{2}, 10 mM MES/KOH pH 5.8 (medium B) followed by gentle centrifugation (50 x g, 5 min). The pellet was resuspended in 0.3 ml medium B, presence of protoplasts or miniprotoplasts was checked by microscopy and flavonoids were extracted by the addition of 0.2 ml resuspended cells to 0.2 ml MeOH. After 2 h at -20° C, samples were centrifuged for 10 min at 18’000 x g and the supernatant was subjected to HPLC analysis. In order to compare different isolations, 10 µl aliquots were taken from the
remaining cell suspension in medium B, added to 1 ml of 80 % acetone and total chlorophyll content was determined by spectrophotometric measurement of absorbances at 470, 647 and 663 nm using the equations of (Lichtenthaler, 1987).

Two different naringenin feeding conditions were used for the demonstration of vacuolar flavonoid transport reactivation following naringenin treatment of ant310 without significant differences in their effectivity. (i) For standard long-term naringenin incubation (2-3 h), 50 µM naringenin (control: solvent only) was present in all media during the preparation of leaf segments, cell wall digestion and protoplast isolation up to the moment of vacuole lysis. (ii) Alternatively, only purified protoplasts were incubated in the presence or absence of naringenin. In order to avoid cell wall regeneration which would subsequently complicate vacuole isolation, medium A was supplied with 0.5 ‰ cellulase Y-C and 0.05 ‰ pectolyase Y-23. In all cases, flavonoid transport experiments were performed immediately after vacuole isolation.

For vacuole uptake studies, naringenin-feeding experiments and corresponding controls were in all cases performed in parallel resulting in a minimum of two independent protoplast and vacuole isolations on the same day.

**Flavonoid compartmentation studies, marker-based assessment of evacuolated miniprotoplasts, and uptake experiments with plant vacuoles**

Vacuolar compartmentation of saponarin reconstituted during naringenin-incubation of ant310 protoplasts (3 h) was analyzed by comparing the amount of saponarin in protoplasts and vacuoles isolated from the same naringenin-treated protoplast preparation. In order to compare both fractions, the activity of the acid phosphatase was determined as a vacuolar marker according to (Hörtensteiner et al., 1992) using methylumbelliferyl-phosphate as a substrate. Fluorescence emission of liberated methylumbelliferone was measured with a Fusion Universal Microplate Analyzer (Packard) and the following filters: \( \lambda_{\text{exc}} = 380 \pm 20 \text{ nm} \); \( \lambda_{\text{em}} = 485 \text{ nm} \). The calculation of the amount of product formed was performed using a methylumbelliferone standard curve. Likewise, absence of vacuolar marker enzymes in evacuolated miniprotoplasts was assessed by measuring the activities of the acid phosphatase, \( \beta \)-N-acetylglucosaminidase and \( \alpha \)-mannosidase in protoplast and miniprotoplast fractions using the corresponding methylumbelliferyl-conjugates as substrates (final concentration 2.4 mM).

Flavonoid uptake experiments into vacuoles isolated from ant310 leaves or protoplasts, that were incubated either in the presence or absence of naringenin, were
performed in parallel using the silicone oil centrifugation technique as described previously (Klein et al., 1996; Frangne et al., 2002). In all cases, six 0.4 ml polyethylene tubes were prepared per condition and time-point containing 5 mM MgATP, 0.1 µCi ³H₂O per tube and 0.1 mM unlabelled isovitexin and saponarin as substrates, which is close to the $K_m$ value of saponarin transport (Frangne et al., 2002). The supernatants of two tubes containing the vacuolar content following centrifugation of the vacuoles through the silicone oil were pooled resulting in 100 µl of which 50 µl were injected for HPLC analysis of the amount of flavonoids transported into vacuoles while 10 µl were subjected to scintillation counting for the calculation of vacuolar volumes. If not stated otherwise, the flavonoid uptake rates were calculated by subtracting values obtained after 18 min of transport from corresponding 3 min values.

**HPLC analysis of flavonoids**

The flavonoid composition of methanolic extracts of leaf segments, protoplasts, miniprotoplasts and vacuoles was analyzed by high performance liquid chromatography (HPLC) performed identically either on a Shimadzu (Japan; experiments performed in Cologne) or on a Gynkotheek (Switzerland; Zurich) HPLC system using the following reverse phase conditions: Nucleosil 100-5 C18 column (125 x 4.6 mm; Macherey-Nagel, Germany); constant flow rate of 1 ml min⁻¹; solvent A = H₂O / 1 % (v/v) H₃PO₄, solvent B = acetonitrile; gradient (B over A; all changes linear) 0-1 min 10-10 %, 1-16 min 10-14 %, 16-26 min 14-14 %, 26-41 min 14-22 %, 41-42 min 22-100 %, 42-43 min 100-10 %, 43-48 min 10-10 %; detection at 315 nm. The Gynkotheek HPLC system was connected to a Dionex (Switzerland) DAD diode array detector. Peaks were therefore identified by co-elution with authentic standards and due to identity of absorption spectra (220 – 370 nm).

**Glucosyltransferase assay.**

The activity of the uridine-diphosphate-glucose: isovitexin 7-O-glucosyltransferase was measured in crude extracts as follows: Segments of 4-5 d old Ca33787 and ant310 primary leaves were incubated in the absence or presence of 50 µM naringenin for 4 h as described above. Liquid N₂-frozen samples were pulverized in a mortar and extracted with 6.25 ml per g FW of extraction buffer (0.1 M KPi pH 7.5, 0.5 mM DTE supplied with 0.2 g Polyclar AT and 0.2 g Dowex Cl⁻ per g FW) for 15 min on ice. The homogenate was filtered through Miracloth and centrifuged (4° C, 20 min, 15’000 x g). The supernatant was purified on a PD-10 column which was previously equilibrated with extraction buffer and used immediately for the activity assay. 10 to 50 µl of extract were incubated at 30° C in 0.1 M KPi pH 7.5, 0.5 mM DTE, 0.4 mg BSA, 3mM UDP-
glucose and 0.2 mM isovitexin in a total volume of 0.1 ml. Assays were stopped by the addition of 1 Vol. MeOH / 1 % (v/v) HCl, centrifuged, and the amount of saponarin formed from isovitexin was analyzed by HPLC. The glucosyltransferase activity was linear over time for at least 60 min and for the protein concentrations up to 45 µg per assay used.
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Figure 1. Scheme of the biosynthesis and compartmentalisation of saponarin in barley and the effect of the ant310 mutation.

Naringenin is formed by the condensation of 3 molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA via the chalcone synthase (CHS) followed by ring closure catalysed by chalcone isomerase (CHI). The following steps of flavone formation towards isovitexin (apigenin 6-C-glucoside) have not been demonstrated in barley up to now and are adapted from Fagopyrum esculentum (Kerscher and Franz, 1987, 1988) and marked by a grey background. Isovitexin is detected in barley leaf extracts in low amounts and is processed to saponarin via an UDP-glucose: isovitexin 7-O-glucosyltransferase (OGT) which is soluble and cytosolic. OGT activity does not appear to be rate-limiting (Blume et al., 1979). Finally, saponarin (and isovitexin) are transported into the vacuole via a flavone glucoside/H⁺-antiporter which is energized by the pH gradient across the tonoplast generated by the activity of the two vacuolar proton pumps, the H⁺-pumping ATPase and pyrophosphatase. The ant310 mutation (italics) causes absence of CHI leading to the formation of a novel substance, isosalipurposide (boxed). glc, glucose; CGT, UDP-glc dependent C-glucosyltransferase; OGT, UDP-glc-O-glucosyltransferases. DIOX, dioxygenase; FNS, flavone synthase (hypothetical).

Figure 2. Naringenin incubation of ant310 leaf segments fully reconstitutes saponarin biosynthesis.

4 d old primary leaf segments of ant310 were incubated in the absence (triangles) or presence of 50 (filled black circles) or 100 µM (open circles) naringenin for the indicated times. Saponarin content in Ca33787 leaf segments without naringenin (grey squares) are depicted as a control. Saponarin content was measured by HPLC.

Figure 3. Naringenin incubation of ant310 mesophyll protoplasts leads to cell-autonomous synthesis of saponarin.

ant310 mesophyll protoplasts isolated from 7 d old primary leaves lacking flavonoids were incubated with or without 50 µM naringenin (filled and open circles, respectively) in the light for the times indicated. Saponarin content in triplicate samples was
determined by HPLC and is expressed based on the chlorophyll content measured in the same sample.

**Figure 4. The presence of the vacuole as a ‘destination compartment’ is necessary for saponarin reconstitution.**

*ant310* mesophyll protoplasts from 7 d old primary leaves (open symbols) and evacuolated miniprotoplasts (filled symbols) were incubated with 50 µM naringenin. Depicted is the reconstituted saponarin content based on chlorophyll after different times of naringenin incubation.

**Figure 5. Inhibitor sensitivity of saponarin biosynthesis reconstitution by naringenin in ant310 protoplasts.**

Isolated *ant310* protoplasts were incubated in the absence (control set to 100%) or presence of the inhibitors indicated together with 50 µM naringenin for 5 h at room temperature before protoplast-associated saponarin content was measured by HPLC. As a further control, naringenin-incubation of protoplasts on ice was analyzed (cold). NH₄Cl was added at a final concentration of 5 mM while CCCP and valinomycin concentrations were 10 µM.

**Figure 6. Naringenin incubation of ant310 leaf segments or protoplasts reactivates the vacuolar flavone glucoside transport activity.**

Vacuoles isolated from 7d old *ant310* primary leaves incubated in the absence (A) or presence (B) of 50 µM naringenin for 3 h were subjected to transport experiments in the presence of 5 mM MgATP and 100 µM isovitexin for 16 min. Depicted are representative HPLC traces where comparable amounts of vacuole extracts were injected. Isovitexin (peak 1) was only taken up into vacuoles after naringenin preincubation of leaf segments (B). Peak 2 designates the *ant310*-specific compound isosalipurposide. (C, D) Quantification of vacuolar transport experiments with 100 µM saponarin (C, time dependency) and isovitexin (D, uptake after 16 min) as substrates. Open symbols/bar: Vacuoles isolated from untreated *ant310* leaves. Filled symbols/bar: Vacuoles from *ant310* leaves incubated with naringenin. Efficient flavonoid transport reoccurs after treatment of leaf segments or protoplasts with naringenin. vv, vacuolar volume.
Figure 7. The reactivation of the vacuolar saponarin transport activity is not immediate.

Protoplasts isolated from 7 d old ant310 leaves were in parallel incubated in the absence (control) or presence of 50 µM naringenin for 10 or 120 min as indicated followed by immediate vacuole isolation and saponarin uptake experiment.
### Table I.

Activity of the UDP-glucose: isovitexin 7-0-glucosyltransferase in 5 d old primary leaf segments incubated for 4 h in the absence (-) or presence (+) of 50 µM naringenin.

<table>
<thead>
<tr>
<th>Line</th>
<th>Naringenin incubation</th>
<th>Activity [pmol saponarin * (min * mg FW)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca33787</td>
<td>-</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>ant310</td>
<td>-</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>ant310</td>
<td>+</td>
<td>2.1 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 2

Graph showing the change in µmol saponarin * g FW⁻¹ over time of incubation (h) from 0 to 7 hours.
Figure 3

The graph illustrates the change in nmol saponarin per mg chlorophyll over time (t) of incubation. The data points are represented by black circles with error bars, showing the variability in nmol saponarin per mg chlorophyll at different time points. The line represents a trend observed over the course of incubation, indicating a possible increase in nmol saponarin per mg chlorophyll as time progresses.
Figure 5

The graph shows the effect of different treatments on saponarin biosynthesis (% of control). The treatments include cycloheximide (µg/ml), NH4Cl, CCCP, valinomycin, and cold. The graph indicates a decrease in saponarin biosynthesis as the concentration of cycloheximide increases, and an increase in biosynthesis when treated with NH4Cl, CCCP, valinomycin, and cold.
Figure 7

$10^{-6}$ mol saponarin x (min x l vv)$^{-1}$

- Control
- 10 min
- 20 min

+ naringenin