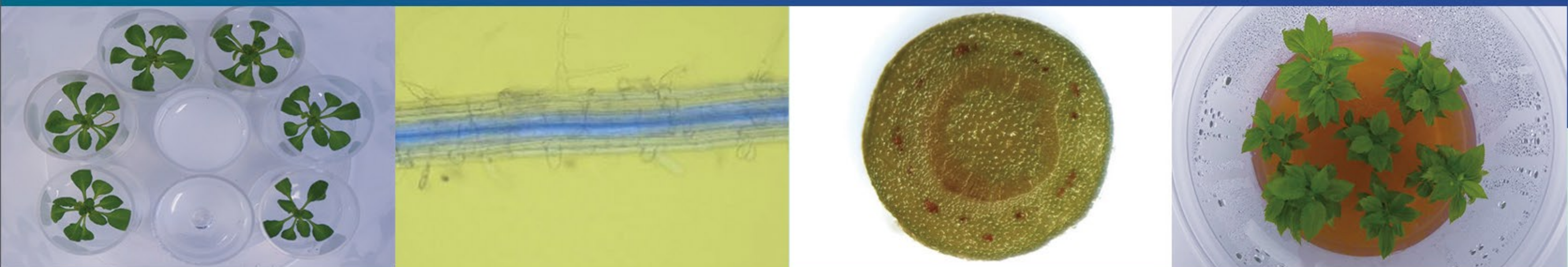
Nutrients translocation & plant growth in tissue culture



Huayi Li

Propositions

1. High humidity does not block transpiration in culture vessels.
   (this thesis)
2. Water translocation is involved in exogenous sucrose utilization in culture.
   (this thesis)
3. Anti-globalization threatens the global economy.
4. Plants will have great performance in agriculture when hydrogen oxidizing bacteria and non-symbiotic nitrogen-fixing bacteria are supplied to the soils.
5. The heart of happiness is self-definition instead of property possession.
6. A society or a person unaware of updated innovation and technology development will be a scary one rather than a brave one.

Propositions belonging to the thesis, entitled

'Nutrients translocation & plant growth in tissue culture'

Huayi Li

Wageningen, 08 April 2020

Nutrients translocation & plant growth in tissue culture

Huayi Li

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Nutrients translocation & plant growth in tissue culture

Huayi Li

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

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To The Great Chinese

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General introduction

Plant tissue culture

Plant tissue culture is the science about the utilization of plant materials, cells, tissues and organs, to propagate and manipulate true-to-type plant clones on an artificial medium in tubes or containers. Although multiplication in tissue culture has been successful in plants of many genera and species, there are still genera, species and even genotypes within them, which are (very) recalcitrant to propagation in tissue culture. Tissue culture often refers to all kinds of *in vitro* plant cultures: culture of organised tissues and culture of unorganised tissues. Organised forms of growth should be created & maintained when tissues containing growing points are transferred in culture such as meristems or shoots. Organised culture allows the maintenance of already existing structures such as young growing points, flower buds and leaf initials. Unorganised tissue culture is used to describe unorganised aggregates of cells such as callus cultures and suspension cultures. To get a substantial growth and multiplication efficiency, the optimization of culture conditions is very important, next to healthy and vigorous initial explants and a suitable medium composed of relevant nutrients.

Explants

The small pieces of tissues which are taken from stock plants and used as starting material are called explants. Any part of the plant can be used as explant but the choice of the most suitable one is important for establishment and maintenance in tissue culture and varies per plant species. Explants suitable for tissue culture should be derived from healthy, disease free and vigorous mother plants (Litz and Conover, 1977; Preece and Read, 2001) that are growing without any stress. The genetic background and physiology of mother plants have a tremendous impact on explant growth and development. Mother plants that have received sufficient energy and nutrients are sources of explants that give a high rate of success in culture. The tissue culture environment is favourable for growth of pests and micro-organisms which might be adhered to or contained inside the explants. Therefore, explants must be treated under aseptic conditions with sterilised tools but prior to that disinfecting chemicals are utilized to kill any micro-organisms that might be present, especially on the outside of the explants. The point is to kill contaminants but with minimal damage to the living plant tissues. The best time for harvesting explants is associated with culture type, plant genotype, explant vitality, season and level of contamination.

Medium

The medium on which plant tissues are grown is supplied with macro and micro elements to maintain sustainable growth of plants. Generally, the medium can be looked upon as a mixture of nutrients, such as amino acids, vitamins, carbohydrates and growth regulators. The morphology of tissues can be greatly affected by medium composition, particularly plant hormones (Irvine et al., 1983; Kim et al., 2003) and nitrate-ammonium salts (Zimmerman, 1981; Piagnani and Eccher, 1987). The media can be provided in two forms, i.e. liquid and solidified by a gelling agent, such as agar or gelrite. Both medium types enable the supply and uptake by explants or plantlets of the necessary nutritional elements. In this study the focus was on solid medium, which was present in cultures in simple glass or plastic containers with relatively little space inside. Plant materials grown on solid medium are generally placed on top of or slightly pressed into the medium and, therefore, have a relative small contact area with the medium and the nutrients therein.

The rate of growth might be slow because, on the one hand, toxic exudates diffuse out and disperse slowly in solid medium and on the other hand, delivery of medium nutrients to the roots might be poor due to the same poor diffusion rates in solidified medium. The advantage of liquid medium is a fast growth rate probably due to the large surface area that is in contact with the nutrients and diffusion rates are generally higher. However, a disadvantage of liquid culture where shoots are submerged, is the risk of hyperhydricity. This can to some extent be overcome by temporary immersion systems (Etienne and Berthouly, 2002).

To promote tissue growth, it would be better to adapt the medium composition to the specific kind of culture and to particular plant genera. The latter has been investigated, however, the effect was too little and at present, the reality is that many plant cultures at commercial scale are grown on very similar media and it seems that a very simple medium "sugar plus mineral salts" is all that is required for micropropagation in many plant species. Still, specific media for specific plant genera are commercially available. Finding the optimal tissue culture medium composition for propagation of your species or cultivar of interest is still a highly empirical process.

Plantlet micropropagation

Micropropagation is the term used to describe propagating plant materials by generating true-to-type clones in which the plants obtained from tissue culture are called microplants. The microplants might be derived from pre-existing shoot buds or meristems, or from unorganised tissues via organogenesis, or somatic embryo formation. In numerous cases, to obtain complete plants, the micropropagated microplants have to be induced to proliferate roots in a later stage prior to transferring to *ex vitro* conditions.

Generally, plant proliferation can be achieved through three propagation methods: direct axillary branching, direct organogenesis (including direct adventitious shoot initiation and direct embryogenesis) and indirect organogenesis (including indirect adventitious shoots, and indirectly initiated somatic embryos from callus). For commercial plant propagation the most reliable and widely applied method is axillary bud/shoot proliferation. Explants are often selected bearing intact shoot meristems, meristem tips, floral meristems or single or multiple nodes. Plants can be easily multiplied through direct axillary branching. Initial explants can develop into a cluster of microplants through axillary bud/shoot culture. Shoot tip cultures are started from meristem segments of main shoots or of lateral shoots from growing plants. Axillary node culture is dependent on stimulating precocious axillary bud outgrowth, which can be relatively easy in many species. Axillary node culture has been successfully set up in species such as *Wrightia tomentosa* (Joshi et al., 2009), *Terminalia bellerica* (Rathore et al., 2008) and *Pinus massoniana* (Zhu et al., 2010). However, since propagation methods as such are not the concern of this thesis I refer to the many studies which have been performed on this topic (a.o. George et al. 2008a and 2008b).

Temporary immersion bioreactors (TIBs) represent a useful tool for plant micropropagation. Continuous tissue proliferation causes nutrient exhaustion of the growth media, requiring regular transfers to fresh medium, often after cutting, which is a large part of the whole production cost (Chu, 1995). Therefore, ensuring sufficient supply and uptake of nutrients are important for labour cost control in tissue culture. In TIB systems, the circulation and uptake of nutrients are relatively easy. Generally speaking,

solid culture allows relative high gas exchange rates while liquid culture makes nutrient uptake easier. The advantage of temporary immersion is the combined high aeration level and high nutrient assimilation rate (Etienne and Berthouly, 2002). Plant growth, morphogenesis and multiplication rates in TIBs are likely better than when plants are cultured on solidified medium or in continuous liquid bioreactors (Berthouly et al., 1995). The positive effects of temporary immersion include aspects of shoot proliferation, production of somatic embryos and survival of acclimatization (Alvard et al., 1993; Etienne et al., 1997; Etienne and Berthouly, 2002). For plant growth and propagation two parameters are the most critical factors in TIB which are the duration of microplant immersion and immersion frequency (Etienne et al., 1997; Etienne and Berthouly, 2002), since they strongly and directly affect water content and water potential in plants, which are important causes of hyperhydricity (Debergh et al., 1981; Ziv, 1994). As for large-scale propagation, important factors determining the success of temporary immersion systems are the simplicity of the bioreactor systems and low facility costs that permit industrialized production at a large scale (Etienne and Berthouly, 2002; Mehrotra et al., 2007). I personally did not investigate nor use TIBs but I keep strong interest in it for the reasons mentioned above.

The production of somatic embryos is a very useful biotechnological tool in plant micropropagation because somatic embryogenesis has a high potential for large-scale propagation generating large numbers of embryos that are generated from somatic cells or tissues (Park et al., 1998). Somatic embryo production is very valuable because it is regarded as a useful tool in genetic manipulation. Somatic embryogenesis can also be employed to shorten a breeding cycle and to conserve endangered species (Okere and Adegeye, 2011; Hussain et al., 2012). In many cases somatic embryogenesis is utilized combined with liquid culture techniques (Ibaraki, 2001; Aitken-Christie et al., 2013).

Photoautotrophic propagation is another micropropagation type in which sugar-free medium is used and the growth of cultures is completely dependent on photosynthesis instead of exogenously supplied sucrose (Kozai et al., 1991; Aitken-Christie et al., 2013). Disorders in aspects of morphogenesis and physiology, such as hyperhydricity are reduced in photoautotrophic propagation. The wide application of photoautotrophic propagation is still limited because of the use of complex technology and relative difficult expertise to regulate the appropriate *in vitro* conditions allowing photosynthesis (Long, 1997), such as the control of light quality, the supply of cooling and the regulation of carbon dioxide. Promotion of net photosynthetic rates of plants as well as growth requires the understanding and subsequent improvement of the necessary environmental conditions. These are not the focus in this thesis but instead we targeted mechanisms of exogenous sucrose and water uptake from the medium and translocation to the apical parts in plants.

For successful plantlet propagation all materials should be sterile and all operations should be performed in an aseptic environment. The environment seems easy: the medium and tools should be autoclaved and cutting and planting are done in flow cabinets. The plant materials might be more difficult because of endogenous microbes inside the plants. Explants from specific organs or parts (hairy, multi-layered or in contact with soil) are also difficult to decontaminate. Bacterial and virus contamination are recommended to be assessed prior to explant establishment. Micropropagation generally knows two stages: the establishment of an aseptic culture and the maintenance of a continuously propagating culture. The selection of proper stock plants without

disease and of the proper aseptic culture conditions are essential for culture establishment. For maintenance of propagation the proliferated plants should be able to be multiplied continuously and these propagated plants should be capable to produce roots to become intact plants which are finally transferred to soil. The objective of the maintenance stage is to produce high numbers of plantlets. These propagated plants should be able to be cultured again (multiplied) to produce a new series of microplants hereby increasing the total number of plantlets produced. Moreover, the maintenance of propagation should go hand-in-hand with guaranteeing high quality of the propagated plants. The promotion of growth of propagated plants is the main target of this thesis.

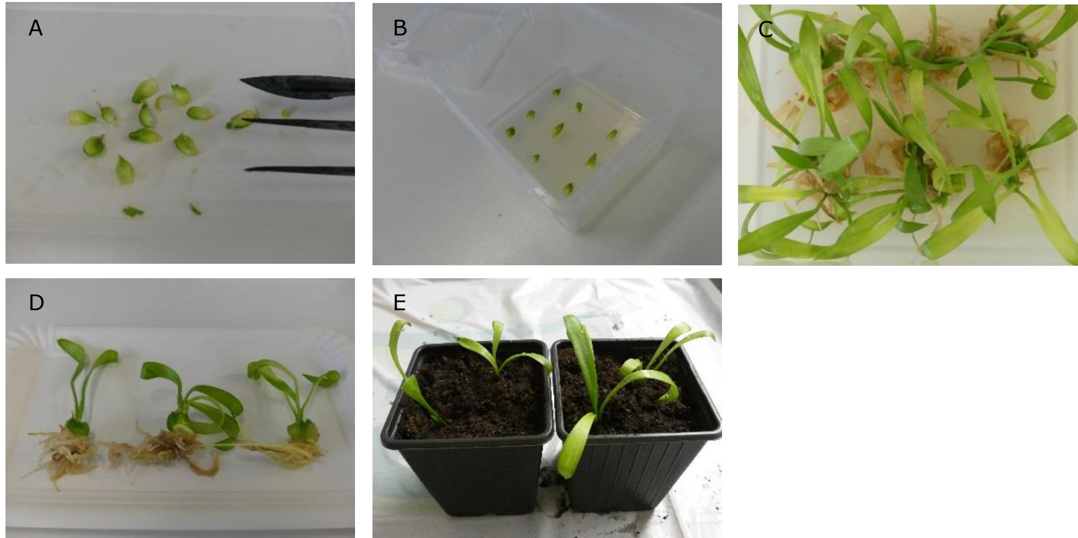

Figure 1. The establishment and maintenance of an aseptic, continuous propagating plantlet culture system as exemplified by *in vitro Lilium longiflorum* cv. 'Snow Queen' propagation. (A) The selected starting explants (scales) in preparation for culture establishment. (B) Initiation of the culture. (C) Bulb production during multiplication. (D) Rooting *in vitro*. (E) Transfer of plantlets to the soil *ex vitro.*

Advantages of tissue culture

First, efficient propagation in tissue culture allows rapid but also large-scale plant material proliferation. Multiple microplants are produced from a single plant in culture. Explants, often small in size, are used to initiate cultures and shoots or tissues also relatively small in size are propagated at an exponential speed in consecutive cultures. This means that only a relatively limited amount of space is needed to accommodate the increasing numbers of propagated shoots. Millions of micropropagated plants can be produced per year (Zhao et al., 1999). Second, infection by specific viruses can be avoided or they can even be removed during *in vitro* culture. Certified, virus-free microplants can be produced in large numbers (Raju and Trolinger, 1986). To remove or avoid pathogens, meristem culture is a general and routine procedure (Cordeiro et al., 2003; Danci et al., 2012; Sastry and Zitter, 2014). The propagation of plants *in vitro* is generally less dependent on seasonal changes. Hence, as third advantage, plants can be produced all year round. Together with cold storage techniques plants can be produced in peak marketing periods (Zhao et al., 1999). Fourth, some plant species are very difficult to be propagated *ex vitro* vegetatively. In that case, tissue culture might provide an

alternative for producing propagule clones. Finally newly selected varieties with high market demand can be produced quickly and in sufficient numbers through micropropagation.

Constraints of tissue culture

It is not possible to ensure that all microplants will survive in culture when plants are transferred to fresh medium or acclimatized for transfer to *ex vitro* conditions. The multiplication rate of cultured tissues normally declines with passage of time. The cultured shoots decrease or lose the capability and viability to form new shoots after a distinct number of cycles of proliferation of high quality shoots (Barnes, 1985; Mendes et al., 1999; Naik et al., 2003). Generally, when establishing an *in vitro* propagation culture, only a relatively low number of explants originating from identical organs survive to start a sustained propagation cycle. However, it is important to keep in mind that explants from the same mother plant but taken at a different physiological age or ontogenetic age or with a different degree of differentiation may not produce the same number and the same quality of propagated clones.

Microbial contamination is frequently encountered in commercial operations (Young et al., 1984; Skirvin et al., 1999) and can lead to substantial losses, rendering months of work worthless (Hussain et al., 1994). However, completely avoiding contamination is a challenge for the industry when thousands of cultures are handled every day. Endogenous contaminations might be invisible at first and pop up at later stages giving un-surmountable problems (Cassells, 1991; Jena and Samal, 2011; Ali et al., 2018). Contamination caused by endophytic microbes cannot be completely stopped with only surface sterilization as the potential production costs and damage to the explants would be too high. On the other hand, the presence of endophytic micro-organisms can be beneficial for plants in *in vitro* culture as was found in apple (Tamosiune et al. 2018).

In some cases, tissues and organs derived from particular plant species might have problems with explant establishment and microplant propagation due to the production of toxic phenolic compounds. The tissues become progressively brown and black, and finally die. The difficult-to-control factor is wounding of the tissue, due to cutting or other reasons, inducing high levels of oxidation and phenolic compounds. Antioxidants, like citric acid and ascorbic acid, and adsorbents like activated charcoal, are being used to stop the oxidization process and the exudation of toxic phenolics.

Another disadvantage of tissue culture is the high costs associated with this technique. Ensuring the presence of the proper facilities and of properly skilled personnel requires a lot of money. Facilities include downflow and crossflow benches and rooms, autoclaves, climate chambers with condition parameters controlled automatically. The current tissue culture procedures make plant production labour intensive and the majority of the budget is spent on manual labour (Ahloowalia and Savangikar, 2004). Medium preparation, container washing, autoclaving, and acclimatization are also labour intensive (Kozai, 2008) although some work can be done in large batches and by machines. Today, the plants are manually selected and cut because *in vitro* produced plants are generally weak and vulnerable and they sometimes have undesirable features such as short epidermal hairs (Zobayed et al., 2001a), poor defined palisade and spongy mesophyll layers (Zobayed et al., 2001b), translucent and fragile leaves (Ziv, 1991), and thick and brittle stems (Park et al., 2004). However, efforts are ongoing to further robotize more and

more steps in the entire procedure including picking and cutting of the microplants. Robotization and automation in plant tissue culture can reduce labour costs which as already mentioned accounts for a high portion of the total production costs (Rowe, 1986; Kozai, 1991a). Robotic techniques are able to replace some of the simpler tasks done by humans but the more advanced human tasks are still quite challenging for robots. However a fair amount of technical training of the personnel involved in the operation is required in order to get properly skilled staff which will make robotization in the long run unavoidable.

Microplants derived from tissue culture are not fully photosynthetically active and are not capable of sustaining the appropriate water balance themselves, when transferred to *ex vitro* conditions. Organic matter is not yet self-produced at sufficient levels and, therefore, plants have to undergo a transitional phase before being transferred to greenhouse or field. Plants derived from *in vitro* conditions should adapt first in a humidity-controlled environment. Here, they become hardened with properly functioning stomata. Stimulating photosynthesis prior to transfer will also help to prepare the microplants for the transition to the field (Lee et al., 1985; Grout and Price, 1987; Kozai, 1991b).

The growth of micropropagated plantlets

Heterotrophic plants in culture might use light to convert inorganic carbon into sucrose by photosynthesis. However, this synthesized sucrose is not enough to sustain growth; exogenous sucrose is generally taken up by plants from the medium in conventional plant micropropagation. That means propagated plants in containers partly or completely rely on this exogenous sugar. Any treatment that improves sucrose assimilation or translocation might promote plant growth and micropropagation efficiency. Research reports on the promotion of sucrose translocation in plantlets in *in vitro* containers are rather scarce. We investigated the relation between growth and sucrose translocation on the one hand and leaf transpiration on the other hand *in vitro*. The level of transpiration in conventional micropropagation is supposed to be very low and the uptake rate of minerals thought to be very inefficient (Afreen, 2005). Generally, *in vitro* transpiration might be promoted via a reduction in the relative humidity in the headspace, stimulating high gas exchange in containers, and probably via activating stomatal conductance. Plant transpiration is influenced by the water balance in the environment inside the container by means of medium evaporation, water condensation on the container inner surface and humidity of the culture room. The *in vitro* relative humidity has a significant impact on the morphology of plants as well as on their growth (Sallanon and Maziere, 1992). The high humidity in the container is mainly due to two factors: restricted gas exchange between inside and outside air, and high water evaporation from the medium. Increasing gas exchange rates in a container might reduce humidity and enhance transpiration. Higher ventilation might lead to increased transport of medium salts as well as organic nutrients. Manipulating relative humidity in a container may improve the quality of plants and growth (Smith et al., 1990). In this thesis the effects on growth of stimulating gas exchange rates and of actively lowering the humidity in the container are assessed.

There are also some other factors that have profound effects on growth. These factors are not the main concern in this thesis but still deserve more investigation. Among these , one factor often ignored is the gelling agent (Debergh, 1983). Heat accumulation at the bottom of the container and hindrance of oxygen diffusion in the medium are believed to be associated with gelling agent type (Tulecke and Nickell, 1959; Bhattacharya et al.,

1994). Less costly gelling agents have been tested such as carrageenan (Bromke and Furiga, 1991), alginate (Johansson, 1988) and a mixture of starch, potato powder and semolina (Prakash et al., 2004). However a reasonably low cost but easy-utilized gelling agent, particularly useful in commercial applications has not been found (Bhattacharya et al., 1994). Another factor influencing growth is the type of culture container, in particular where it affects relative humidity (Böttcher et al., 1988), gas accumulation or diffusion (Gavinlertvatana et al., 1982) and plant water retention strength (Debergh et al., 1981).

Transpiration

Transpiration is widely believed to be at a very low level *in vitro* and subsequently the water flow is supposed to be marginal but there is very little scientific evidence for this (Tanaka et al., 1992). Important factors that are involved in transpiration of *in vitro* plants include low gas exchange between container and culture room, sealed closure of containers, and high humidity in the headspace (Fuchigami et al., 1981; Shackel et al., 1990; Tanaka et al., 1992). In principle, transpiration can take place from the epidermal cells through the cuticle or from the sub-stomatal cavities through the stomata (Figure 2). Environmental and endogenous factors manipulate stomatal movements, such as light colour (Kinoshita et al., 2001; Sothern et al., 2002), ABA (Tal, 1966; Ko et al., 2006), cytokinins and auxins (Tanaka et al., 2006), accumulated ethylene in the container (Tanaka et al., 2005), differences in CO_2 concentration between ambient air and the inside of the container (Hashimoto et al., 2006; Teng et al., 2006; Young et al., 2006), humidity (Assmann et al., 2000; Xie et al., 2006), and various abiotic stresses (Hetherington and Woodward, 2003). Plasma membrane H^+-ATPase and K^+ channels regulate stomatal opening/closing, influenced by shifts in symplastic osmotic potential which result from water flux in guard cells (Humble and Raschke, 1971; Schroeder, 1988; Roelfsema and Hedrich, 2005). Water loss is also determined by the water permeability of the cuticle, albeit to a lesser extent. The leaf surfaces in vascular plants are covered with a matrix of polymeric cutin, the cuticle layer (Goodwin and Jenks, 2005). Cuticle transpiration can vary under the influence of physical thickness, polymeric structure and chemical composition (Riederer and Schreiber, 2001; Burghardt and Riederer, 2008).

Plant growth *in vitro* might be associated with the level of transpiration. It is not known to what extent biomass increase or growth *in vitro* are related to the level of transpiration and what factors exert an influence on this. As reported, insufficient transpiration might bring about problems of un-remobilised element transport, e.g. of calcium in lettuce, and of necrosis at leaf tips *in vitro* (Collier and Huntington, 1983). The supply of dissolved minerals and their distribution *in vitro* are determined by the flow of water (Williams, 1993). In order to optimize plantlet growth *in vitro* and facilitate acclimatization upon transfer to soil, the internal aerial humidity inside the container should be reduced to a lower level (Brainerd and Fuchigami, 1981; Ziv et al., 1982; Preece and Sutter, 1991). Vapour pressure gradients increasing plantlet transpiration might be improved by utilization of bottom cooling of shelves and highly permeable filters (Vanderschaeghe and Debergh, 1987; Ghashghaie et al., 1992).

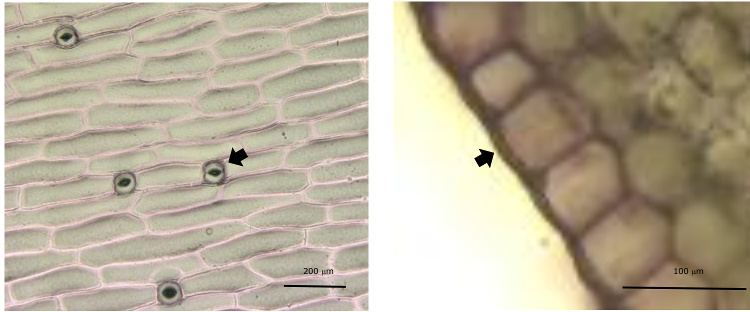

Figure 2. Stomata (shown by arrowhead in the left) on a bulb scale of *Lilium Oriental* hybrid cv. 'Santander' grown *in vitro* and the cuticle (shown by the arrowhead in purple staining on the right) on the adaxial side of a bulb scale of *Lilium Oriental* hybrid cv. 'Santander' grown *in vitro*.

Water transport

For plant growth and development one of the most important physiological aspects is the absorption and transport of both water and nutrient elements. For the overall water transport at the whole plant level, water goes passively along gradients in water pressure, called the water potential gradients (Steudle, 1994; Schultz, 2001). The mechanism of overall water movement all the way to the top of a plant has been established, the cohesion-tension theory (Bohm, 1893), although alternative mechanisms have been put forward provoking a debate (Zimmermann et al., 1994; Canny, 1998; Zimmermann et al., 2004). It is supposed that water tension in the xylem system (negative pressure) as a consequence of leaf transpiration is involved in water movement to the plant's most upper aerial parts (Steudle, 2001). The water potential gradient and the transpirational pull from continuous water columns in the vascular tissues might stimulate the inflow of water and dissolved nutrients from the medium via roots and finally into leaves *in vitro*.

Generally water and nutrients are absorbed by the youngest parts (Tsukagoshi and Shinohara, 2016) and root hairs in roots. Roots of propagated plants represent an important link in the medium-plant-air continuum (Resh, 2016), as a pathway for water translocation, resembling the soil-plant-air continuum *in vivo*. After being taken up, water goes across the epidermis and passes a series of concentric cell layers. Before finally arriving at the vascular tissues water has to cross the exodermis, a few cortex cells, endodermis, pericycle, and parenchyma (Steudle and Peterson, 1998). For a radial flow along potential gradients in roots, water has three possibilities: the apoplastic pathway through cell walls and intercellular spaces, the symplastic pathway through plasmodesmata and the transcellular pathway through membranes (Steudle and Peterson, 1998). The apoplastic route through porous cell walls is generally efficient but the Casparian strip, which is composed of deposited lignin and suberin, and which is present in cell walls in exodermal and endodermal cells acts as a strong apoplastic barrier to the flow of water and solutes (Tester and Leigh, 2001). Therefore, water and nutrient solutes have to enter the cell-to-cell pathway at this point, crossing membranes. The plasma membrane bound water channel proteins, called aquaporins or PIPs might be involved in determining membrane water flux, also in *in vitro* roots. Aquaporin-rich or aquaporin-active membranes may promote water flux. But the contribution of aquaporin is dependent on age of roots, developmental stage, hydrostatic driving forces and osmotic driving forces. Supposedly, water flow in the apoplastic pathway is driven by hydrostatic forces and in transmembrane pathway is driven by osmotic gradient forces

(Steudle, 1994; Steudle and Peterson, 1998). The quantitative contribution of the symplastic pathway via plasmodesmata to the water flow is not known (Passioura, 1988; Zhang and Tyerman, 1991). Finally water goes upwards axially once it has arrived at the root vascular system and the water, if not used, transpires via leaves as vapour.

Sucrose transport

The establishment of a successful plant tissue culture procedure is greatly influenced by the composition of the propagation medium, particularly with respect to the sugar utilized which has an important role in maintaining plant growth (Rosa et al., 2009), acting as nutrient and process regulator (Sheen et al., 1999; Smeekens, 2000; Rolland and Sheen, 2005), affecting morphogenesis and shoot regeneration (Brown et al., 1979), for development and in stress responses (Rolland et al., 2002; Rolland et al., 2006; O'hara et al., 2013). Sucrose, the predominately used carbohydrate *in vitro*, being the sugar present in the phloem sap of plants (Thompson and Thorpe, 1987; Fuentes et al., 2000; Ahmad et al., 2007), is generally considered as the best sugar, meeting energy demands and growth requirements *in vitro.* However, the response can vary with species and culture conditions (Thompson and Thorpe, 1987; Fotopoulos and Sotiropoulos, 2004). Still, few investigations have been performed on how this exogenous carbohydrate is taken up and translocated *in vitro* (Jain et al., 1997). The transport of medium nutrients, including sucrose, *in vitro* is considered to be suboptimal because transpiration as the driving force for water and dissolved nutrient flow is supposed to be at a low level (Kozai and Kubota, 2005).

For an *ex vitro* plant sugars are delivered over a long distance via a source-to-sink pathway which is a major determinant of plant growth. It is important to understand the sugar delivery process from the source cells where carbon is fixed photosynthetically and subsequently exported to the sink cells where sugar is partitioned and transiently stored in the vacuole. Symplastic phloem loading, apoplastic loading and polymer trapping are the mechanisms by which sucrose is delivered and collected into the phloem (Van Bel, 1993; Rennie and Turgeon, 2009; Slewinski and Braun, 2010). In the symplastic pathway, sucrose moves down a concentration gradient from mesophyll cell to phloem sieve elements. The symplastic sucrose movement is a passive loading system and energy is not required there (Rennie and Turgeon, 2009; Slewinski and Braun, 2010). The number of plasmodesmata connecting companion cells of the sieve elements to surrounding cells might become limiting at some point in time and, as an alternative, sucrose might enter the apoplastic pathway (Evert et al., 1978; Haritatos et al., 2000; Braun and Slewinski, 2009). This requires export of sucrose through the plasma membrane of phloem parenchyma cells and off-loading into the apoplast; later, sucrose needs to be uploaded again into the companion cells-sieve elements and enter the phloem (Chen et al., 2012). The translocation of sucrose into the phloem takes place against a concentration gradient and, therefore, requires energy and finally leads to high levels of sucrose in the sieve tubes (Geiger et al., 1973; Giaquinta, 1983; Winter et al., 1992; Berthier et al., 2009). After long distance transport, sucrose is finally unloaded in sink sites either symplastically, or apoplastically or through a combination of both (Oparka, 1990; Patrick, 1997; Zhang et al., 2006; Ma et al., 2018; Milne et al., 2018).

The actual translocation of exogenously supplied sucrose, in either radial route or axial direction, in plants *in vitro* in medium-plant-atmosphere continuum is hardly investigated. The available literature shows that the supply of exogenous sucrose to cultured plants

impaired photosynthetic biochemical reactions (Fuentes et al., 2005; Lobo et al., 2015), such as PSII activity and Rubisco abundance (Lobo et al., 2015), and sucrose phosphate synthase activity (Van Le et al., 2001). More research exploring different angles are required to reveal details on the mechanism of uptake and translocation of exogenous sucrose in *in vitro* propagation.

Growth enhancement potential

The success of plant tissue growth in culture depends on the uptake and utilization of medium elements. Applied nutrients, minerals and carbohydrates for instance, are amply available in media. The point is whether the level of nutrient uptake and transport is sufficient or rather suboptimal and whether it is possible to optimize tissue growth and take development to a higher level *in vitro* by stimulating these processes.

In most cases, microplants are cultured on a static medium where the wound surface and lower parts are in direct contact with the nutrients in the medium. Exceptions are culture in liquid medium or temporary immersion systems where entire plants are in contact with the culture medium. On solidified media, the organs and tissues in contact often have inadequate surface areas where nutrients can be taken up. In most cases sugar will be taken up at a restricted region, the small epidermal area and the cut surface, at the medium-plantlet contact interface (Mengel, 1984). No studies so far, however, were focussed on the possible process of nutrient assimilation by cultured shoots (Guan and De Klerk, 2000; George et al., 2008a). Nutrient gradients between the medium and the tissue in contact might be relatively high and the diffusion rate of nutrients in the medium, necessary for replenishing the assimilated nutrients, might be relatively low. The exudation and accumulation of deleterious by-products of plant metabolism might also interfere with the process of nutrient uptake *in vitro* and as a consequence with plant growth.

The internal microclimate greatly influences plant development because in most cases culture parameters in containers might be far from optimal, including parameters of irradiance intensity, temperature, and photoperiod for specific cultivars in particular culture stages. It is general practice to optimize the culture room environment in order to obtain plants of high quality. To optimize plant growth, also the container environment should be finely regulated to accommodate the best possible growth of particular plant species at a commercial scale. The light irradiance that can be given in tissue culture is usually rather low (Chauvin and Salesses, 1987), but the light demand can be much higher depending on the plant species (Evers, 1984). However, high light irradiance will bring about all kinds of other problems (Fink et al., 1986; Hazarika, 2006). The type of container covers and walls are associated with light transmittance (Fujiwara et al., 1989; Huang and Chen, 2005) affecting light irradiance and spectra that reach the microplant. The distance between fluorescent tubes and containers and their spacing have a big impact on shoot multiplication via irradiance reduction and spread (Chen, 2005). Important wavelengths for photomorphogenesis are 400–800 nm while light at other wavelengths are still necessary; however certain wavelengths cannot pass the container wall boundary, e.g. in polycarbonate those shorter than 390 nm, and in glass those shorter than 290 nm (Dooley, 1991; Nhut et al., 2000; Moon et al., 2006). However, the authors did not use different containers or different light conditions to investigate plant growth; emphasis was put on the importance of container and light. Apart from wavelength and light flux density the duration of light exposure also influences *in vitro*

growth and morphogenesis (Hillman, 1961; Pavlová and Krekule, 1984; Morini et al., 1991). The growth is partly proportional to the length of time when plants can photosynthesize. Plants are able to recognise changes in light duration and have a shift in morphogenetic response. The length of the photoperiod is modified to manipulate growth and morphogenesis in case of preparation of qualified stock plants from which explants will be excised (George et al., 2008b). Another physical factor is the temperature which is also vital to manipulate cultured tissues. When commercial demand for plants decreases, the temperature can be altered to suspend growth (Hausman et al., 1994; Trejgell et al., 2015), therefore reducing the costs of tissue culturing (Janeiro et al., 1995). High temperature might interrupt cytokinin induced shoot formation (Fonnesbech, 1974; Nievola et al., 2017). The range of temperature for tissue culture is 20-27 °C in most cases. Manipulating culture conditions, the internal microenvironment and the external environment, by various factors, as a synergic effect, has been widely applied to enhance growth performance *in vitro*.

Scope of the thesis

In this thesis, I aimed to better understand what physiological and molecular genetic factors affect plant growth *in vitro* and how to attempt to promote growth by manipulating such factors. In particular, I undertook studies to assess the importance of transpiration for biomass accumulation under a general high humidity microenvironment in two plant species, a model plant and an actually cultivated crop. I also investigated the processes of water and sucrose absorption and translocation from medium to roots of plants grown in tissue culture at the molecular level. The specific objectives were:

To determine whether transpiration contributes to plant growth *in vitro* although plants are cultured under high humidity conditions;

To understand the impact of both cuticular water loss and stomatal water loss on plant biomass accumulation *in vitr*o;

To define whether and how sucrose transporter genes mediate exogenous sucrose assimilation in roots;

To investigate whether aquaporin genes are associated with water uptake and plant growth *in vitro*;

To establish whether it is possible to develop a method to optimize plant growth by applying stomatal opening inducers;

And finally to study whether impediment of cuticle formation might alter plant growth *in vitro*.

In **Chapter 1** I introduced the plant growth situation in tissue culture and present the effects of various factors on plant growth performance and morphogenesis *in vitro*.

In **Chapter 2** I investigated the determinants of growth *in vitro* by looking at transpiration at the plant level studying the roles of the surface cuticle and of stomatal activity. I found that both transpiration and sucrose assimilation attributed by water transport contributed to biomass accumulation in plants as the main reason for growth. The results showed that it is highly likely that carbohydrate absorption, transportation and distribution are dependent on water influx and efflux, even in high humidity *in vitro*

conditions as shown by using stomata mutants and cuticle mutants of *Arabidopsis thaliana*.

In **Chapter 3** I examined the role of sucrose transporter genes in the process of exogenous sucrose translocation via apoplast and transmembrane routes in roots. I looked at the gene expression levels of SWEET and SUC genes in leaves and roots in both *ex vitro* as well as *in vitro* conditions, determined carbohydrate levels biochemically, and studied growth performance by measuring fresh and dry weights. GUS analysis revealed that the translocator genes SWEET11 and SWEET12 were induced by sucrose available in the medium. The isolated root culture assay showed that knocking out both SWEET11 and SWEET12 leads to a decrease in sugar uptake in roots. Esculin fluorescence was studied in mesophyll protoplasts and root cell protoplasts of *sweet11;12* in comparison to wild type. The results demonstrated the importance of SWEET11 and SWEET12 genes in exogenous sucrose assimilation and translocation *in vitro*.

In **Chapter 4** I emphasized the contribution of aquaporin plasma membrane intrinsic proteins PIP1 to the growth of *in vitro* plants through investigating physiological characteristics in miRNA-induced-PIP1s-silenced plants. The results showed that aquaporin PIP1s were low in expression and induced by sucrose in the medium. Plants silenced in PIP1s expression showed reduced growth and reduced biomass accumulation but more sucrose accumulated in roots *in vitro*. Water loss from leaves as a measure of transpiration was monitored but no difference was observed between the wild type and the PIP1s-silenced plants *in vitro*. The outcome of the experiments confirmed the importance of PIP1s by showing that PIP1s indirectly mediate sucrose amounts in roots and that PIP1s are involved in growth *in vitro*.

In **Chapter 5** I investigated whether increased transpiration generally can lead to improved plantlet growth in shoot culture by using *Malus domestica* 'Gala' and by testing the relationship between transpiration, stomatal and cuticular transpiration, and plantlet growth. Plantlets were tested in containers varying in potential air exchange rates. Acid fuchsine was used to demonstrate the role of transpiration in element migration. Plantlets grown in reduced relative humidity by application of a small vial of hygroscopic potassium chloride exhibited an increased transpiration and an increase in dry weight. Opening the stomata also increased transpiration and biomass accumulation as seen by applying δ-aminolevulinic acid to the medium. I also investigated plantlet growth after inhibiting cuticle formation using metolachlor and chemical solvents. In summary, plantlet growth was associated with transpiration which could be manipulated in various ways *in vitro*.

In **Chapter 6** I combine the results from the experimental chapters and discuss the implications for understanding plant growth in tissue culture. The processes of water & sucrose uptake and transport by the *in vitro* plants are described and tentatively explained. Using these new results the current state of plant tissue culture and its future prospects are discussed.

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CHAPTER 2

Determination of transpiration of plantlets growing *in vitro.* Role of stomata and cuticle.

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Abstract

The growth performance of plantlets *in vitro* is dependent on their capability to absorb and translocate nutrients, such as sucrose, from the culture medium. The medium in this case represents the carbohydrate source, while the developing plantlet's parts and organs represent the sink. Many factors in the artificial culture environment, which is far from being optimal, may influence the growth performance of *in vitro* plantlets. Our aim is to find options for stimulating biomass accumulation and plantlet growth *in vitro*. The physiological research presented here was mainly focussed on the transpiration of *in vitro* plantlets as a driving force of water and nutrient translocation. In particular two aspects were investigated, i.e. cuticle functionality and stomatal conductance. The contribution of transpiration through the cuticle of leaves *in vitro* has been studied, a.o. using mutant *Arabidopsis thaliana* lines with a damaged wax deposition, such as *cer5* and impaired cuticle formation, such as *att1*. The mutant lines showed enhanced water loss and increased cuticle permeation as well. *In vitro, att1* showed increased fresh weight and dry weight levels while *cer5* did not. Apart from this, a stomata mutant Arabidopsis line, *epf1epf2* having a higher density of stomata, also showed improved growth *in vitro* while the stomata mutant line *spch* with fewer stomata, did not. Our research revealed that a significant increase of water loss from leaves was linked to the increase in stomatal density in *Arabidopsis thaliana*, demonstrating the relevance of stomatal transpiration for growth *in vitro.*

Our results suggest that it is possible to enhance growth and elevate biomass of micropropagated plantlets by regulating the cuticle and/or stomata characteristics to stimulate transpiration through both. Our results also indicate that it will be important to assess whether these findings also hold true for other plant species to improve growth.

Keywords: transpiration, biomass accumulation, stomata, cuticle, micropropagation, nutrient translocation, *Arabidopsis thaliana*

Introduction

With respect to micropropagation there are two main types: photoautotrophic micropropagation in which the carbon and energy source are derived from endogenous photosynthesis, and the other, heterotrophic or photomixotrophic micropropagation in which growth is totally or partly dependent on exogenous sucrose supply in medium (Kozai et al., 2005). In this article we consider plantlets growing in containers on sucrose containing media, hence the photomixotrophic system, which is the one predominantly used in mass *in vitro* multiplication (Rahman and Alsadon, 2007). Micropropagation allows the fast generation of pathogen-free plantlets of genetically uniform background. However, the growth is far from optimal compared to *ex vitro* conditions because of e.g. the internal atmosphere consisting of a water saturated headspace, which has variable and low CO_2 levels, and contains released ethylene and other detrimental gases (Proft et al., 1985; Kozai, 2010a). As a result, this may cause anomalies in plantlet physiological, anatomical and morphological characteristics (Mohamed and Alsadon, 2010; Iarema et al., 2012). Under normal *in vitro* conditions transpiration and water balance of micropropagated plant tissues are considered to be widely impaired because of the high relative humidity inside the containers (Fuchigami et al., 1981; Shackel et al., 1990; Tanaka et al., 1992). It has been reported that in order to promote plantlet growth *in vitro,* transpiration of vapour should be elevated through altering humidity in culture vessels before transplanting (Brainerd and Fuchigami, 1981; Ziv et al., 1982; Preece and Sutter, 1991). In addition to taking external measures to reduce humidity, such as using microboxes equipped with gas-permeable filters in the lids, we investigated possibilities at the plant level for transpiration stimulation and biomass regulation. In principle, transpiration can take place from the epidermal cells through the cuticle or from the sub-stomatal cavities through stomata. Here, we investigated the effects on growth *in vitro* by looking at transpiration at the plant level studying the roles of the surface cuticle and of stomatal activity.

The surface of aerial tissues in vascular plants is covered with a cuticle layer which is composed of a matrix of polymeric cutin (Goodwin and Jenks, 2005). Plant non-stomatal transpiration varies because of cuticle thickness, wax amount, polyester structure and composition (Riederer and Schreiber, 2001; Burghardt and Riederer, 2008). The cuticle is synthesized and secreted by epidermal cells and prevents plants from inappropriate uncontrolled water loss (Kerstiens, 2006; Kosma and Jenks, 2007), dust deposits (Wagner et al., 2003), fungal spore and bacterial infection (Jenks et al., 2002) and UV light radiation and damage (Krauss et al., 1997). However, it is not clear to what extent the cuticle component feature is relevant to biomass accumulation through influencing water flow and carbohydrate translocation *in vitro*.

Cuticle components are synthesized by joining carbon atoms of acetyl-coenzyme A (acetyl-CoA) together into long carbon chain fatty acids. The cuticle formation comprises three main steps. At first, aliphatic acids are synthesized in plastids of epidermal cells. These de novo C_{16} and C_{18} long-chain fatty acids are activated by acyl-CoA synthetase in the cytoplasm (Schnurr and Shockey, 2004; Samuels et al., 2008). The C_{16} and C_{18} fatty acids are ubiquitous intermediates for all lipid synthetic processes (Samuels et al., 2008). In next stage, C_{16} and C_{18} fatty acids are elongated into C_{20} and C_{34} extended chains by enzyme complex elongases in the endoplasmic reticulum (ER) and converted to very-long-chain fatty acid acyl-CoAs (VLCFAs) (Liu and Post-Beittenmiller, 1995; Jenks et al., 2002; Joubès et al., 2008). In the final step of wax synthesis, VLCFAs are modified

yielding compounds with subgroups of alkyl esters, alkanes, alcohols, aldehydes and ketones, and are further mobilized to the plasma membrane followed by being exported to exterior lipid matrix (Samuels et al., 2008; Bernard and Joubès, 2013).

An important gene involved in the cuticle synthesis process is ATT1, an abbreviation for *Aberrant induction of Type Three genes.* ATT1 functions in biosynthesis of cutin-specific, ω-hydroxy and α, ω-dicarboxy fatty acids by encoding a cytochrome P450 fatty acid ω-hydroxylase (CYP86A2, At4g00360) (Xiao et al., 2004; Rupasinghe et al., 2007). Mutant *att1* plants are characterized by a loose cuticle ultrastructure, by having one third of the normal cutin amount, and by showing increased water vapour permeability indicating the role of ATT1 in cuticle development and regulation of cuticular water loss (Xiao et al., 2004; Duan and Schuler, 2005). Apart from this, mutant *att1* also increases expression of the bacterial type III effector gene *avrPto* and the bacterial master regulatory gene *hrpL* both from *Pseudomonas syringae* pv. *phaseolicola* (Xiao et al., 2004; Block et al., 2008). As a consequence, the a*tt1* mutant in *Arabidopsis thaliana* exhibits increased susceptibility to the pathogen *P. syringae*, indicating a role for the wild type gene in disease resistance (Xiao et al., 2004). CER5 (AT1G51500), eceriferum 5 also named as ABCG12, abbreviated for ABC transporter G family member 12, or WBC12, short for white-brown complex 12, is required for wax secretion and transport from epidermal cells to the cuticle surface (Rashotte et al., 2001; Pighin et al., 2004; Panikashvili et al., 2007). CER5 is found expressed exclusively in epidermal cells in plants as shown by GUS activity assays (Pighin et al., 2004), while mutagenesis of the gene results in very bright green stems and glossy siliques (Koornneef et al., 1989). *Cer5* is characterized by features, such as, a reduction of cuticular wax deposition by epidermal cells, accumulated sheet-like inclusions in the cytoplasm, and its phenotype is only detectable in stems and leaves (Pighin et al., 2004). In this investigation we used next to the wild type *Arabidopsis thaliana, att1* and *cer5* mutant lines as study materials.

The gas exchange between the plant's surface and the atmosphere occurs primarily through stomata, which are pores formed by pairs of guard cells. Stomatal characteristics and its activities determine gas exchange and water efflux. It has been discovered that the water use efficiency is regulated, in part, by changes in stomatal density, defined as the number of stomata per unit surface area (Masle et al., 2005). The stomatal density and stomatal opening size concomitantly determine maximum stomatal diffusive conductance constraining maximum gas diffusive capacity (Franks and Beerling, 2009; Franks et al., 2009). Other studies also report that stomatal density substantially affects vapour diffusion (Schlüter et al., 2003; Yoo et al., 2010; Doheny-Adams et al., 2012). However, it is not clear whether this stomatal trait is correlated with biomass accumulation by influencing transpiration and nutrient flow *in vitro*.

Arabidopsis stomata development comprises three main stages characterized by successive progenitor cells. SPEECHLESS (SPCH), MUTE and FAMA are 'master regulator' genes in stomata differentiation which resemble basic helix-loop-helix domain transcription factors (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). SPCH initiates stem cell differentiation through division of undifferentiated epidermal cells, and regulates asymmetric division into stomatal lineage (MacAlister et al., 2007; Pillitteri et al., 2007). MUTE is involved in termination of the asymmetric divisions after meristemoids undergoing several 'amplifying' asymmetric divisions, and promotes differentiation of meristemoids to oval shaped guard mother cell (GMC) (MacAlister et al., 2007; Pillitteri et al., 2007). In the final stage FAMA, MYB88 and FOUR LIPS (FLP) are

required for GMC undergoing equal symmetric division and producing the stomatal opening between guard cell pairs (Lai et al., 2005; Ohashi-Ito and Bergmann, 2006). EPIDERMAL PATTERNING FACTOR 2 (EPF2) is expressed in early precursors, meristemoid mother cells (MMCs) and early meristemoids (Hunt and Gray, 2009), and regulates stomata patterning by preventing protodermal cells from entering meristemoid mother cell fate (Hara et al., 2009; Hunt and Gray, 2009). EPIDERMAL PATTERNING FACTOR 1 (EPF1) is produced and secreted by the meristemoid and its early descendants, and mediates asymmetric cell division in stomatal complex pattern, and regulates stomatal development inhibition (Hara et al., 2007; Hara et al., 2009). In our study we used *spch* and *epf1epf2* mutant lines as study materials.

Water transport and efflux in plantlets might be influenced by an alteration in wax and cutin deposition in the cuticle and by raising the number of stomata. Plantlet growth *in vitro* is probably dependent on nutrition uptake and transport which are driven by water loss or transpiration through the leaf cuticle surface and through the stomata. In our hypothesis, plantlet's growth *in vitro,* being far from satisfactory, is related to transpiration and nutrient migration. Our research goal is to find options for stimulating biomass accumulation and growth performance *in vitro* and to see whether this can be achieved through investigation of cuticular traits and stomatal activity.

Materials and methods

Plant materials and growth conditions

All *Arabidopsis thaliana* lines were in Columbia-0 genetic background and the transgenic T-DNA insertion mutant lines including mutants *att1* (SALK_005826C), *cer5* (SALK_036776), *spch* (SAIL_36_B06) and *epf1epf2* (SALK_137549 and SALK_047918) were obtained from the European Arabidopsis Stock Centre. All seeds were sterilized by immersion in 70% (v/v) ethanol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 1 min followed by 2% (w/v) sodium hypochlorite (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 15 minutes, and rinsed in sterile deionized water three times, and then placed on Petri dishes with full-strength MS media (Duchefa, Haarlem, The Netherlands)(Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin agar (Duchefa, Haarlem, The Netherlands) under darkness at 4 °C for 72 hours to synchronize germination. These germinated seeds were sown under two different growth conditions, either in pots or in rectangular containers. Plants were grown in soil mixture with 2/3 peat (Kekkilä Oy, Äyritie, Finland) and 1/3 vermiculite (Kekkilä Oy, Äyritie, Finland) in the greenhouse; or in rectangular Steri Vent container (107 × 94 × 96 mm)(Duchefa, Haarlem, The Netherlands) containing autoclave-sterilized (121°C for 20 minutes) full-strength MS medium (Duchefa, Haarlem, The Netherlands), 2% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin agar (Duchefa, Haarlem, The Netherlands), pH 5.7-5.8 in a growth chamber (Conviron BDR16)(Conviron Germany GmbH, Berlin, Germany) with as parameters light intensity at 50 μmol m^{-2} s^{-1} (Philips Master TL-D 36)(Philips, Poland), 16 h light and 8 h dark, and temperature 21°C. All seed batches were collected from plants grown in soil pots in the greenhouse under identical growth conditions. The greenhouse condition parameters are temperature 21°C in day time, 19°C at night, 16/8 hour photoperiod, and relative humidity 70%.

Mutant screening assay

Arabidopsis lines after 21 days of growth *in vitro* were screened for expression levels of the appropriate genes using real time PCR. Total RNA was extracted from leaves of various transgenic lines using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Gel electrophoresis was used to confirm RNA quality. RNA was subjected to remove genomic DNA using RNase-free DNase I (Qiagen, Hilden, Germany). The reaction programme was: incubation at 20 °C for 15 min of 10 μL mixture of 1 μg RNA, 1 μL 10X DNaseI buffer plus enzyme; the reaction was stopped by diluting the reaction mixture 1:1 with 11 μL of 25 mM EDTA and incubating at 65 °C for 10 min. The cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The 20 μL reaction mixture containing 11 μL DNase treated RNA, 4 μL 5X iScript reaction buffer, 1 μL iScript reverse transcriptase was incubated in a program being 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, 4°C for 5 min, 85°C for 5 min, and was subsequently kept at 10°C. Synthesized cDNA was diluted 10 times for further use. Expression levels were tested by real-time PCR using primers shown in the supplementary table 1. The amplification was performed using the SYBR Green kit on 7500 PCR system (Bio-Rad Laboratories, Hercules, USA) following manufacturer's instructions. The 10 μL reaction mixture contained 2 μL diluted cDNA, 5 μL 2X SYBR GREEN mix DNA fluorescent dye (Bio-Rad Laboratories, Hercules, USA), 0.3 μL forward/reverse primers. Real-time quantitative PCR

assays were performed as follows: 95°C for 10 min, 1 cycle; 40 cycles of 95°C 15s, 55°C 30s, 72°C 30s; 72°C for 10 min. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using Actin-2 as reference gene. Gel electrophoresis was used to show the amplified real time PCR products.

Cuticle permeability assay

The permeability of the cuticle was determined by using a toluidine blue penetration test. This was done by submerging leaves of *in vitro* grown plants in a 0.1% (w/v) toluidine blue (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) solution for ten minutes. After incubation, leaves were gently washed in running tap water to clear any excess of solution from the surface and subsequently they were photographed (Canon EOS 4000D)(Canon Inc., Tokyo, Japan). Another method to test cuticle permeability was to quantify chlorophyll leakage (Sieber et al., 2000). For this, aboveground parts containing leaves and stems were removed, weighed and immersed in 20 ml of 80% ethanol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) after 500uM ABA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) treatment overnight which closed stomata. The chlorophyll was released in the ethanol in a shaker (IKA-Labortechnik AS 501.4, Staufen, Germany) at a rotation speed of approximately 50 rpm in a dark room. Using a spectrophotometer (UV-VIS-1800 spectrophotometer)(Shimadzu, Suzhou, China), light absorbance was measured for the quantification of chlorophyll under wavelength 664 and 647 nm. Micromolar content of chlorophyll was calculated per unit of fresh weight using formula $7.93\times(A_{664}$ nm$) + 19.53\times(A_{647}$ nm$)$ (Lolle et al., 1997).

Water loss assay

Rosette leaves, collected from *in vitro* grown Arabidopsis mutant lines at the end of the fourth week, were kept at room temperature with the adaxial side down in open Petri dishes on the bench. Leaves from five plants per line were excised, collected and subsequently weighed in petri dishes at time 0. Next, the dishes were weighed using XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands) at the time 30 min after excision. The weight loss represented the water lost through transpiration/evaporation.

Growth measurement

Arabidopsis plants, which were grown in rectangular containers under the same conditions as mentioned earlier, were collected for weight analysis after 4 weeks of growth. Plants were carefully removed from the medium, soaked in water to dispose of medium adhering to them, and gently rubbed dry in tissue paper. These plantlets were subsequently placed in empty tubes of which the weight was already known and were weighted by a 0.01 mg resolution XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands). Subsequently, the plantlets were dried in an oven (Omnilabo International B.V., Breda, The Netherlands) at 70 °C overnight and were then weighed using the 0.01 mg resolution analytical balance again.

Stomatal traits identification

Stomatal features from mature rosette leaves were recorded by imprinting leaves in dental resin (Coltene Whaledent, Raiffeisenstraße, Germany) producing impressions on nail varnish as described by Hepworth et al., (2015). In experiments, stomatal imprints were taken from the abaxial side of mature rosette leaves of 3-week-old plantlets grown

in vitro. The counting of the stomatal impressions was carried out on as an average ten plantlets per Arabidopsis line by using Axiophot light microscope (Carl Zeiss Microscopy, White Plains, United States). The stomatal complex size and density were quantified by using the Image J programme (United States National Institutes of Health and the Laboratory for Optical and Computational Instrumentation in University of Wisconsin, USA)(Gitz and Baker, 2009).

Statistical analysis

The analysis was done between wild type Col-0 and the mutant lines by one-way ANOVA together with Bonferroni-Holm adjustment test or t test using statistical package GraphPad Prism (GraphPad Software, San Diego, USA) or excel. Values were presented as means ±SEM. Asterisks above each bar represent statistically significant differences (*, $P<0.05$ or **, $P<0.01$).

Results

Verification of used mutant lines

Quantitative real time PCR showed ATT1 and CER5 expression levels in lines *att1* and *cer5,* respectively as well as SPCH expression in *spch* and EPF1 and EPF2 expression in *epf1epf2*. The T-DNA insertion mutant *att1* (Figure 1A) had a very low gene expression as well as *cer5* (Figure 1A). Quantitative RT-PCR showed that SPCH, EPF1 and EPF2 expression levels were low in the mutant lines s*pch* and *epf1epf2*, respectively (Figure 1B). The stable levels of Actin2, used as internal control, and the very low expression levels of ATT1 and CER5 are also shown in bands after gel electrophoresis (Figure 1C and 1D). The results confirmed that *att1* and *cer5* mutants had low expression levels of their respective knocked-out genes. The levels of Actin2 (Figure 1E and 1F), were used as internal control and were found to be stable; marginal levels of SPCH, EPF1 and EPF2 were shown in PCR fragments using gel electrophoresis (Figure 1E and 1F). The results proved that s*pch* and *epf1epf2* mutants had marginal expression of genes SPCH, EPF1 and EPF2 respectively.

The role of cuticle *in vitro*

To determine the role of the cuticle in transpiration for growth under *in vitro* conditions we used wild-type *Arabidopsis thaliana* Col-0 and mutant lines impaired in cuticular traits: a*tt1*, having a loose cuticle (Xiao et al., 2004; Duan and Schuler, 2005), and *cer5*, showing ultrastructural reduction of cuticular wax deposition (Pighin et al., 2004). Water loss and biomass increase were evaluated in our research to establish the role of the cuticle in growth *in vitro*.

Cuticle permeability

Cuticle permeability was studied by exposing rosette leaves to an aqueous toluidine blue solution for a short period of time. The extent of toluidine blue penetration in the leaves was monitored and taken as a measure for cuticle permeability. The tests were done with wild type Col-0 *in vitro* plantlets representing the normal scenario and with the two mutants, *att1* and *cer5*, having a seriously impaired cuticle or wax deposition. *Att1* displayed an evenly spread staining in the leaves all over, by contrast, the wild type did not show any purple staining (Figure 2A). The staining pattern displayed by *cer5* was characterized by a more patchy and random staining pattern but the staining intensity was considerably less compared to *att1* (Figure 2A). Another way to test cuticular features is by monitoring permeability through measuring chlorophyll release into ethanol after submersion of leaves. Chlorophyll leakage into alcohol was more prominent in *att1* and *cer5* when compared to wild type Col-0 (Figure 2B).

Water loss

Transpiration from leaves occurs either through the stomata or through the epidermal cuticle. Micrographs of stomatal features were compared in Col-0, *att1* and *cer5 in vitro.* The stomatal number (Figure 3B) and the stomatal size (Figure 3C) were examined but no significant differences were found (Figure 3). Therefore, in our water loss assay the stomatal transpiration of lines Col-0, *att1* and *cer5* was considered to be the same (Figure 3); any differences observed were regarded as due to differences in transpiration through the epidermal cuticle. We measured transpiration, shown by water loss

percentage, by weighing fresh weight half an hour after plantlets were collected and their roots were excised on the bench. The mutant line *att1* displayed the highest water loss of 38%, *cer5* displayed somewhat lower water loss of 35% but the wild type Col-0 differed significantly at 27% (Figure 2C). Visual observations showed that the leaves from the mutant lines had a more wrinkly, wilted appearance compared to Col-0 demonstrating that both cuticle mutants had a higher water transpiration rate in these lab room conditions (data not shown here).

Taken together, the results confirmed that *att1* and *cer5* mutant lines showed altered cuticular traits, and in particular had a higher molecule permeability and enhanced water loss capacity *in vitro*, in short a higher transpiration rate.

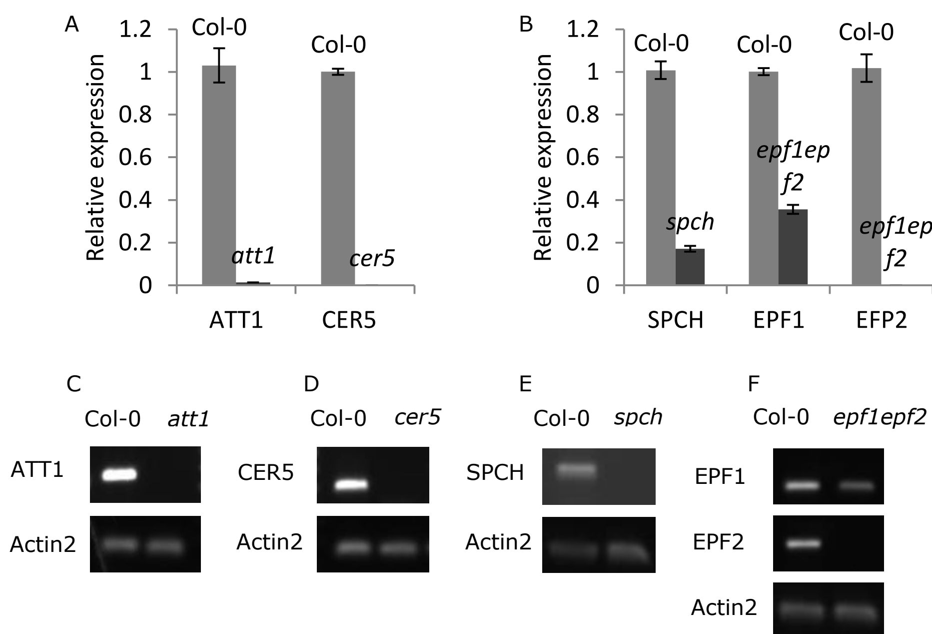
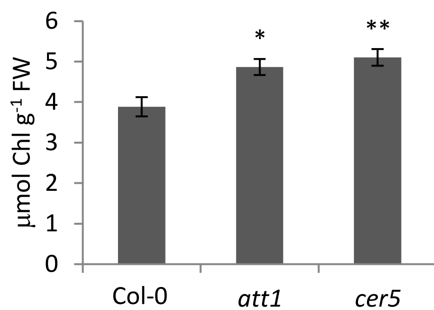


Figure 1. Molecular characterization of the cuticle mutant lines *att1* and *cer5,* and of the stomata mutant lines *spch* and *epf1epf2*. A, RT-PCR testing ATT1 and CER5 gene expression in *att1* and *cer5* compared to wild type Col-0 using housekeeping gene Actin2 as reference. B, RT-PCR testing SPCH, EPF1 and EPF2 gene expression in mutant line *spch* and double mutant line *epf1epf2* compared to wild type Col-0 again using housekeeping gene Actin2 as reference. C, transcript levels for ATT1 in the *att1* mutant line and Col-0 in gel electrophoresis after RT-PCR. D, transcript levels for CER5 in the *cer5* mutant line and Col-0 in gel electrophoresis after RT-PCR. E, the transcript levels for SPCH in the *spch* mutant line and Col-0 in gel electrophoresis after RT-PCR. F, transcript levels for EPF1 and EPF2 in the *epf1epf2* mutant line and Col-0 in gel electrophoresis after RT-PCR. The level of Actin2 expression is also shown.

A

B



C

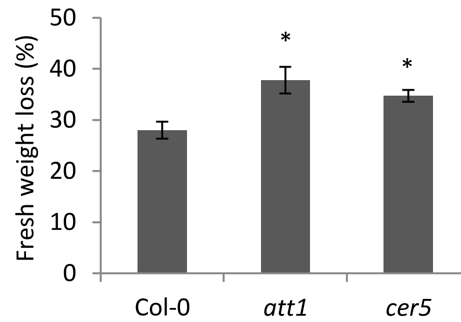


Figure 2. Cuticle permeability and water loss of *in vitro* grown *Arabidopsis thaliana* wild type Col-0 and the mutant lines *att1* and *cer5*. (A) Rosette leaves of Col-0, *att1* and *cer5* respectively stained in toluidine blue for ten minutes. One of the five replicates is shown for each line. (B) Chlorophyll leakage from rosette leaves of Col-0, *att1* and *cer5* respectively (mean ± SEM). Each value represents five biological replicates. (C) Water loss measured at half an hour after rosette leaf excision of Col-0, *att1* and *cer5* respectively (mean ± SEM, n = 15 plants). The experiment was repeated three times with similar results. Asterisks indicate significant differences at P<0.05 (*) or P<0.01 (**).

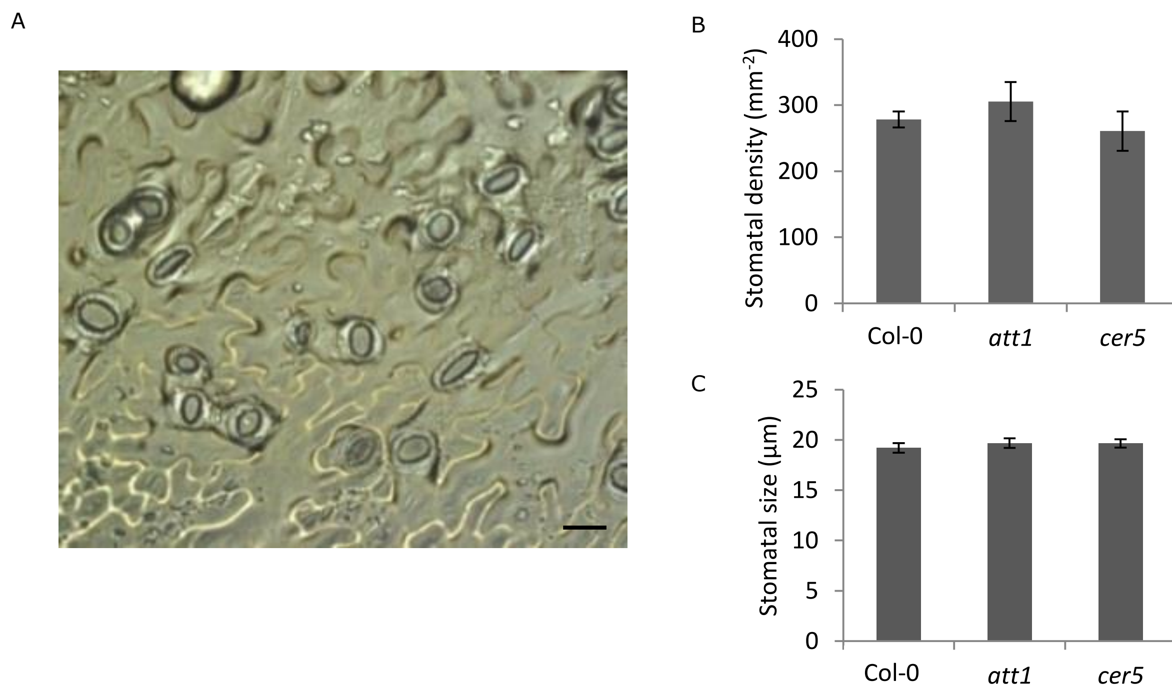


Figure 3. Stomatal features of *Arabidopsis thaliana* leaves from wild type Col-0 and the two mutant lines *att1* and *cer5*. (A) Micrograph of an epidermal peel of the adaxial side of a representative leaf taken from an *in vitro* wildtype plantlet. Scale represents 20 μm. (B) Stomatal density and (C) stomatal size on the abaxial side of leaves from *att1* and *cer5* transgenic lines in comparison to Col-0 (mean ± SEM; n= 10-15 plants per line). No significant differences were found.

Growth and biomass increase *in vitro*

Our hypothesis is that plantlet growth is determined by water flow and concomitant nutrient assimilation. More transpiration, even *in vitro*, might be involved in more extensive plantlet growth and in increased biomass accumulation. The mutants impaired in cuticle properties showed increased leaf permeability and increased water loss (Figure 2). On the other hand, the altered cuticles might have an influence on the plant's plasticity (Kutschera and Niklas, 2007; Savaldi-Goldstein et al., 2007), which could consequently lead to altered plant growth phenotypes. Here, we determined the effect of cuticle alteration on plantlets growth through investigation of fresh weight and general growth profile. The mutant line *cer5* displayed obvious growth retardation and a significant reduction in fresh weight accumulation, while line *att1* showed enhanced growth, both in fresh weight increase as well as visibly in general phenotypic screening (Figures 4A & 4B). Line *att1* exhibited statistically significant higher growth represented by a higher fresh weight of 66.83 mg compared to wild type Col-0, at 39.62 mg; line *cer5*, on the other hand, showed a reduction in fresh weight, 27.51 mg (Figure 4A). Clearly, the two wax mutants have an opposite effect on *in vitro* growth while transpiration seemed stimulated in both.

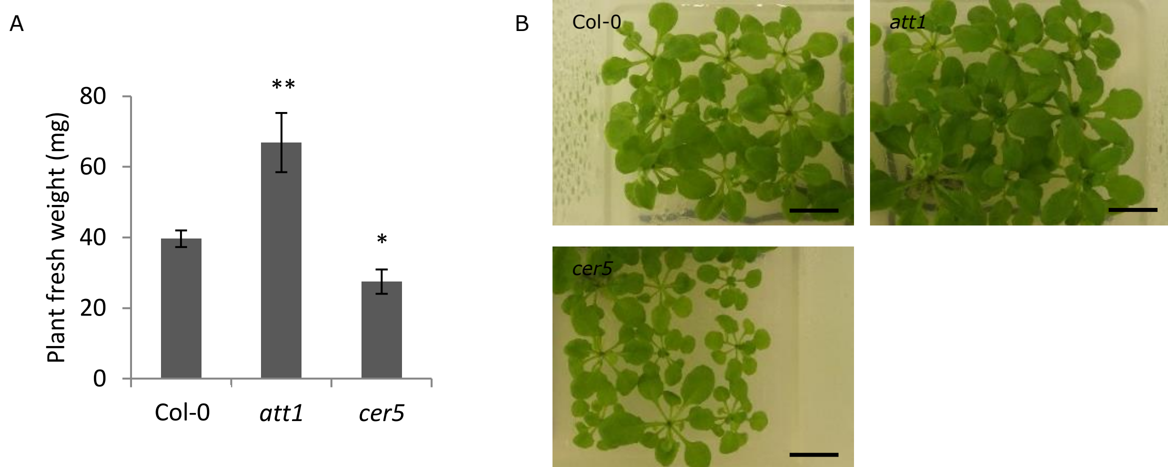


Figure 4. Growth of the cuticle mutants *att1* and *cer5 in vitro* compared to the wild type Col-0 control. (A) Fresh weight of mutant lines *att1* and *cer5* relative to wild type Col-0 after 28 days of growth (mean ± SEM). Each value represents fifteen biological replicates. (B) The morphological phenotype of wild type plants Col-0 and *att1* and *cer5* plants grown *in vitro* for 4 weeks. The scale bars represent 1 cm length. Asterisks indicate significant differences at P<0.05 (*) or P<0.01 (**).

The role of stomata *in vitro*

The water balance of plantlets is regulated by stomatal structure and activity. The stomatal properties from plantlets *in vitro* might have a clear influence on water migration and exogenous sugar assimilation and translocation. Here, we monitored the role stomatal density played in water efflux and biomass accumulation *in vitro* using lines *spch* and *epf1epf2*.

Stomatal density and stomatal aperture size

Stomatal characteristics were investigated in the leaves of *spch, epf1epf2* and wild type Col-0 *in vitro*_grown plants (Figure 5A). The mutant line *epf1epf2* exhibited a higher stomata density, 412.14 mm^{-2}, and *spch* showed quite a low stomata number of 73.35 per mm^{-2} relative to wild type Col-0, 188.33 mm^{-2} on the abaxial side (Figure 5B). The stomatal aperture size was also monitored in our research: a statistically lower aperture size in *epf1epf2*, 4.61 μm, and an unchanged aperture size in *spch*, 5.24 μm, relative to the aperture size in Col-0 of 5.90 μm, was found. These data indicated a possible negative correlation between stomatal pore size and stomatal density in the mutant line *epf1epf2* but not in line *spch*.

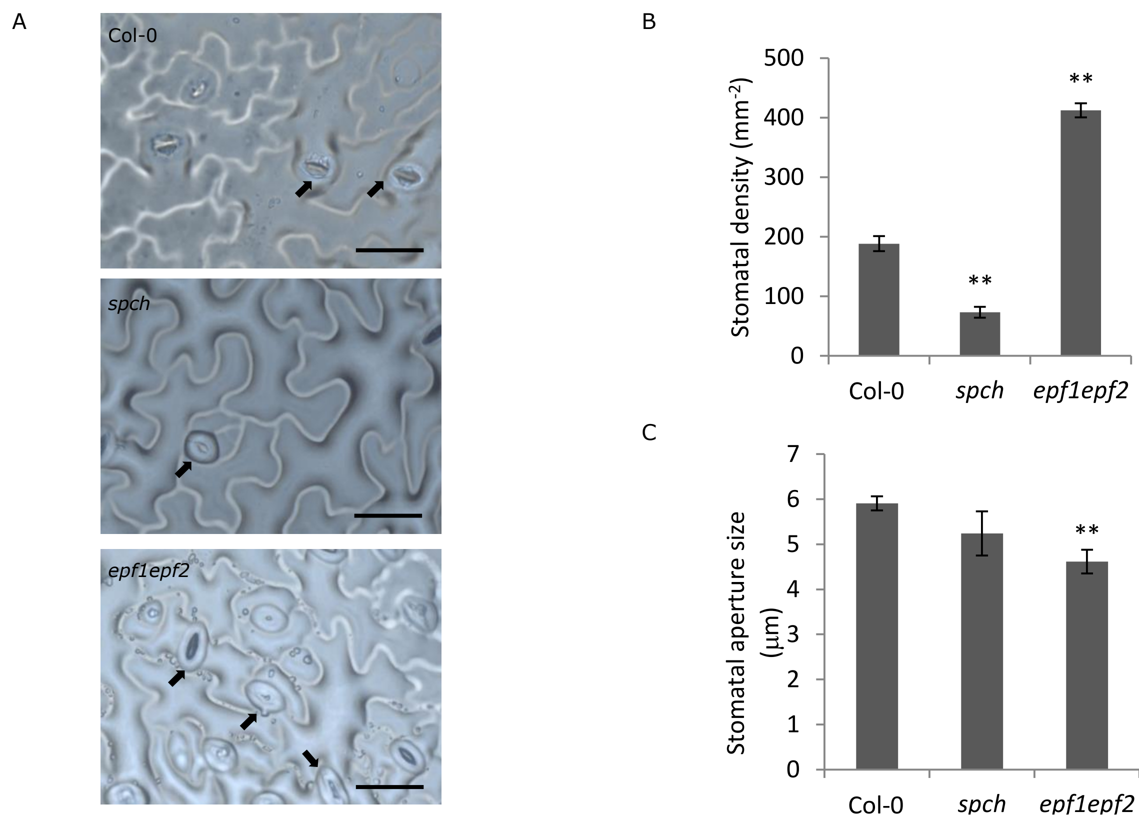


Figure 5. Stomatal characteristics of Arabidopsis mutant lines *spch* and *epf1epf2* grown *in vitro* compared to Col-0. (A) Micrographs of abaxial epidermal peels from wild type, *spch* and *epf1epf2*, respectively. Arrowhead shows site of stomata. (B) Stomatal density in mutant lines *spch* and *epf1epf2* grown *in vitro* (mean ± SEM). (C) Stomatal aperture size in mutant lines *spch* and *epf1epf2* grown *in vitro* (mean ± SEM). Each value represents ten to fifteen biological replications. Scale bars represent 20 μm. Asterisks here indicate significant differences at P<0.01 (**).

Stomatal transpiration and growth

To assess whether stomatal alterations might affect plantlet transpiration and biomass accumulation *in vitro* we conducted water loss assays and growth measurement experiments. The rate of water loss was tested at room temperature and the experimental outcome showed that water loss in *epf1epf2* was enhanced by 35%, 26%, and 22% compared with wild type Col-0 at 30min, 60min and 120min respectively while water loss in *spch* was reduced by 24%, 13% and 5% in 30min, 60min and 120min respectively (Figure 6B). To assess the biomass feature in *spch* and *epf1epf2* we performed gravimetric measurements of fresh weight. *In vitro,* line *epf1epf2* showed increased growth in size (Figure 6A) and exhibited a higher fresh biomass of 55.88 mg compared to Col-0 48.4 mg while *spch* had 29.9 mg fresh weight (Figure 6C) indicating that the stomatal number influenced nutrient translocation and biomass accumulation *in vitro*.

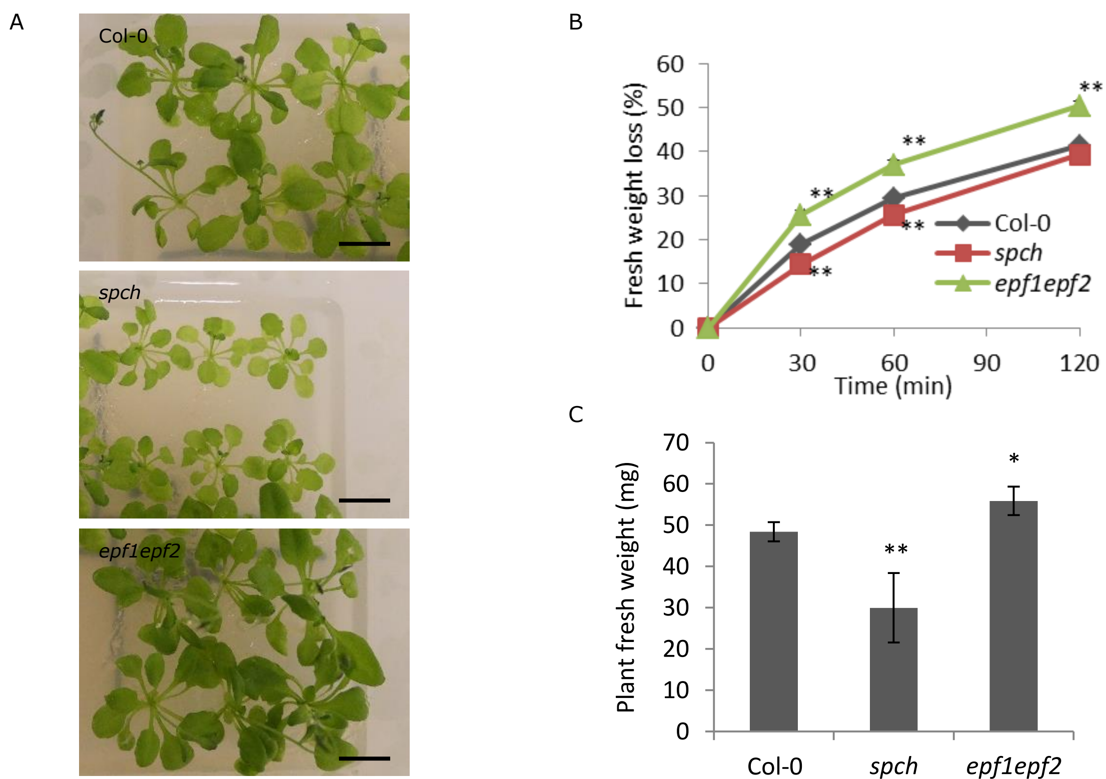


Figure 6. Water loss and growth of *spch* and *epf1epf2* mutants *in vitro*. (A) Plantlet phenotype of *spch* and *epf1epf2* mutants *in vitro* compared to Col-0. (B) Water loss measured in detached rosette leaves of *spch* and *epf1epf2* mutants *in vitro* at different time intervals compared to Col-0 (mean ± SEM). Each value represents ten to fifteen biological replications. (C) Fresh weight of *spch* and *epf1epf2* mutant lines relative to wild type Col-0 (mean ± SEM). Each value represents fifteen biological replications. All scale bars represent 1 cm length. Asterisks indicate significant differences at $P<0.05$ (*) or $P<0.01$ (**).

Comparison of cuticle and stomata effects on growth *in vitro*

To assess whether the cuticle or the stomata contributed more to biomass increase of microplants *in vitro* the dry weights of cuticle mutant lines *att1* and *cer5*, and stomata mutant lines *spch* and *epf1epf2*, were determined in one experiment. Line *cer5* showed 41% reduced dry biomass while line *att1* revealed 71% increased dry biomass compared to Col-0. Meanwhile mutant *spch* with fewer stomata, showed a 65% reduction in dry biomass, while *epf1epf2*, with a doubled stomata density, showed a 30% increase of dry weight compared to the wild type. *Att1* and *epf1epf2* both showed increased transpiration and enhanced growth, albeit that *att1* grew significantly better than *epf1epf2*, while the percentage water loss (transpiration) was at a similar level with 35% for *att1* and 34% for *epf1epf2*. However, these transpiration results did not come from the same experiment. *Spch* showed less transpiration and a concomitant decrease in growth; only *cer5* deviated having more transpiration but lower growth.

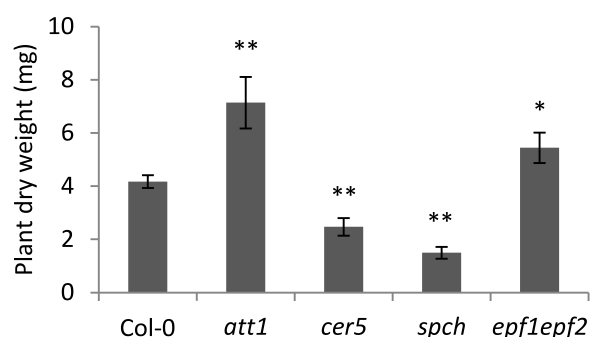


Figure 7. Dry biomass comparison of cuticle mutant lines *att1* and *cer5*, and stomata mutant lines *spch* and *epf1epf2 in vitro* relative to wild type Col-0 (mean ± SEM). Each value represents ten to fifteen biological replications. Asterisks indicate significant differences at P<0.05 (*) or P<0.01 (**).

Discussion

Conventional *in vitro* propagation is widely used for multiplication of both economically important plant species and some rare species. It is a widely-used route for plantlet production because it allows fast reproduction of a large quantity of disease free plantlets in the same genetic background (George et al., 2008). Micropropagation has been extensively studied and reported but investigations on factors determining plantlet growth *in vitro* are rare. Most of the research is performed on the effects of gelling agents, sugar composition, plant growth regulators and the optimization of physical micropropagation conditions (Debergh and Read, 1991; Kozai et al., 1992; Jeong et al., 1995; Krikorian, 1995). The closed aerial environment influences plantlet's growth and is responsible for factors like high relative humidity, low light irradiance, potential ethylene accumulation and unstable carbon dioxide concentration (Zimmerman, 1994; Aitken-Christie et al., 2013). The growth characteristics, from an anatomical, morphological or physiological angle, often exhibit anomalies, such as hooked, curled or fragile leaves, low in strength, poorly generated shoots, new shoots with fewer biomass and peculiar trichomes (Kozai et al., 2010b; Cha-um et al., 2011; Xiao et al., 2011).

The water balance is greatly influenced by physiological processes, nutrient quantity and the number of plantlets present within a culture vessel (Murashige and Skoog, 1962). The leaf tip necrosis of lettuce grown *in vitro* was correlated to a deficiency of calcium which was attributed to limited transpiration in high humidity vessel (Collier and Huntington, 1983). Transpiration and liquid flow determine the uptake and transport of nutrients such as carbohydrates and ions *in vitro* (Williams, 1993). We hypothesized that transpiration and sugar flow contribute to carbohydrate assimilation and accumulation of microplants. The microplant physiological features could be improved through lowering internal relative humidity and enhancing plantlet transpiration in the closed *in vitro* system (Ghashghaie et al., 1992; Sallanon and Maziere, 1992; Tanaka et al., 1992). To ameliorate plantlet growth two techniques have been used to increase the vapour pressure gradient, and herewith reduce the relative humidity *in vitro*: bottom cooling and the utilization of lids featured with high water vapour permeable filters (Vanderschaeghe and Debergh, 1987; Ghashghaie et al., 1992). Apart from creating optimized external conditions beneficial for plant transpiration, water transport driven by transpiration can be promoted intrinsically within plants, e.g through cuticle surface alteration and stomata manipulation. This study investigated the correlation in micropropagated plantlets between water loss as indicator for transpiration and growth capacity, focussing on alterations in cuticle functionality or stomatal density, using the appropriate Arabidopsis mutant lines.

The role of cuticle transpiration in growth *in vitro*

1 Cuticle permeability

To investigate cuticle properties we monitored cuticle permeability by staining leaves after submersion in an aqueous cationic dye, toluidine blue, and by measuring chlorophyll leakage in ethanol. In these two experimental systems, the toluidine blue assay measures inward permeability (Tanaka et al., 2004) and the chlorophyll leakage assay measures outward permeability (Aharoni et al., 2004). Any role of the stomata in this was excluded by the ABA treatment given to the plants prior to the penetration tests. The *cer5* mutant, which was characterized by an impaired secretion process of cuticular wax from epidermal cells to the cuticle (Pighin et al., 2004), showed staining of very

small areas of leaves in a patchy pattern. Earlier Tanaka et al. (2004) showed staining but restraint to petiole regions (Tanaka et al., 2004). In our hands, the leaves of the *att1* mutant, which was reported to have reduced biosynthesis of hydroxylated fatty acids which are required for cutin biosynthesis and cuticle development (Xiao et al., 2004; Rupasinghe et al., 2007), appeared much more permeable showing intensive toluidine blue staining. Hence, it can be concluded that the two cuticle mutants differed in inward permeability. Chlorophyll leakage was higher in *cer5* and *att1* than in Col-0, suggesting a difference in outward permeability, too, but the other way round. Permeability in measurements are indicators for cuticular integrity. The cuticular integrity in both mutants appeared to be affected in nonpolar route and in polar route. It is still not clear how cuticular integrity and permeability features in our mutant lines are affected in details. The used experimental setups just gave indications about permeability-related features but could not discriminate in cuticle quantitative or qualitative chemical or physical variation.

The cuticle plays a fundamental role in regulation of the water balance between the plant epidermal surface and ambient air. The weight loss of excised leaves in *att1* and *cer5* was tested in thirty minutes at room temperature in the laboratory: a*tt1* and *cer5* exhibited higher water loss than wild type. Actually the plant's cuticle is characterized as a very thin layer, of which the thickness ranges from 1 μm to 15 μm, and of which the inner layer faces the apoplastic aqueous phase (Schreiber and Schonherr, 2009). Aqueous solutions may penetrate the cuticle in two directions, outwards and inwards, but the predominant flow direction might be outwards as the ambient humidity is generally lower than leaves interior. This process is known as cuticular transpiration (Schreiber and Schonherr, 2009). Previously, researchers attempted to establish a universal correlation between cuticle thickness and cuticular transpiration, but failed (Schönherr, 1982; Schreiber and Riederer, 1996; Sampangi-Ramaiah et al., 2016). However, the key role of the cuticle as a water barrier is clear: cuticle permeability increases 100 fold more after wax extraction (Schreiber, 2010) and a reduction in cutin increases transpiration significantly in the *cutin deficient1* Arabidopsis mutant (Isaacson et al., 2009).

As the stomatal pores also determine water loss we investigated whether a change in cuticle permeability as observed in the mutant lines was of influence on stomatal traits. It was reported that variation in cuticle integrity may affect stomatal features (Gray et al., 2000; Chen et al., 2003; Aharoni et al., 2004). Hence, stomatal density and stomatal size were determined on the abaxial side of leaves via resin imprints. However, it seemed that in our mutants guard cell development was not altered as shown in Figure 2. It indicated ATT1 and CER5 were not involved in guard cell and epidermal cell development in *Arabidopsis thaliana* and that stomatal conductance might be similar in the mutants to the wild type Col-0, meaning that any difference that was observed is due to cuticle transpiration and not to stomatal transpiration. Besides it is good to keep in mind that measuring physiological parameters *in situ*, such as total transpiration, relative humidity, stomatal and cuticle conductance is not technically possible in the closed *in vitro* containers without interfering or disturbing the growth environment and risking contamination.

A further detailed biochemical study of the cuticle properties in composition and structure could help to unravel the relationship between the biochemical traits and permeability and cuticular transpiration. Still, our results implied that altering cuticle integrity as in *att1* and *cer5* had strongly influenced permeability as visualized by dye penetration,

mesophyll chlorophyll leakage and increased water loss. This showed that even in closed containers highly saturated with water, the transpiration through the cuticle did occur in *in vitro* grown *Arabidopsis* seedlings.

2 Sucrose assimilation and biomass accumulation *in vitro*

Minerals and carbohydrates are available and even abundant in any artificial propagation medium whereas nutrient assimilation and translocation processes might be insufficient to support optimal growth *in vitro*. The success of plant tissue culture is greatly affected by nutrient, sucrose for instance, assimilation and utilization. Nutrient acquisition takes place through medium-plant contact at interface layers in tissue culture. Unlike plants *ex vitro* where the inorganic solution is assimilated through root hairs and symbiotic mycorrhizae, nutrient acquisition *in vitro*, organic and inorganic, is restricted to the small epidermal and small cut surface area at the plant-medium interface (Mengel, 1984). Although the nutrient assimilation *in vitro* and *in vivo* are likely to be very different in the physiological and biochemical processes, there could be similarities in certain aspects of them. So far, no studies on uptake of nutrients in *in vitro* shoot cultures have been performed (El-Ramady et al., 2014).

Sucrose is considered as the predominant carbohydrate in tissue propagation and the most generally present sugar in phloem sap of micropropagated plants (Thompson and Thorpe, 1987; Fuentes et al., 2000; Ahmad et al., 2007). This carbohydrate is considered as the only carbon source which is to meet growth requirements *in vitro* but the demand for it depends upon culture conditions and might vary according to species (Thompson and Thorpe, 1987; Fotopoulos and Sotiropoulos, 2004; Singh and Kumar, 2009). Nevertheless, little research has focussed on the mechanism and process of carbohydrate acquisition and absorption *in vitro* (Jain et al., 1997). The hypothesis is that the sugar uptake *in vitro* is driven via active absorption which is affected by cation excretion, by a liquid flow which requires free water, and through passive diffusion which is associated with concentration gradients (Komor et al., 1977; Komor et al., 1981). Nutrient uptake under *in vitro* conditions is considered to be low because transpiration as driving force for liquid flow is there but generally insufficient to allow nutrient translocation in a way comparable to the normal, *ex vitro* situation (Kozai and Kubota, 2001). Increasing transpiration, e.g. cuticular transpiration, could promote nutrient movement upwards and may improve plantlet's growth *in vitro* (Roberts et al., 1994). Transgenic line *att1* showed impaired cuticle integrity and improved water loss in previous sections. This complies with greater sensitivity to dehydration, easier wilt leaves, higher epidermal permeability in *att1 ex vitro* (Xiao et al., 2004). Higher transpiration *in vitro*, we postulate, stimulates nutrient translocation upwards, and therefore results in higher biomass in *att1*. This transpiration alteration is thought related with change in extracellular lipids in leaves, interesterification of hydroxy fatty acids in cutin, and total cutin monomers (Xiao et al., 2004). The wax in a hydrophobic film covers plant surface and damage in wax might remove transpiration limitation in *cer5*. The total wax load for cer5 leaves has been significantly lower than wild type, not only primary alcohol is reduced but also ester level is lower respectively, of which primary alcohol reduction is due to reduced constituents of C_{26} and C_{28} (Rashotte et al., 2001). Line *cer5* which was impaired in epidermal cuticular wax secretion (Pighin et al., 2004), did show enhanced cuticular transpiration but unexpectedly showed a reduction in growth *in vitro*. The reduced growth in *cer5* implies that the growth might be negatively related to process of blocked wax secretion and transport from epidermal cells to the cuticle surface and, more

importantly, not all increases in cuticular transpiration automatically have improved growth--other unknown factors also might influence growth *in vitro*.

In fact water permeability at the cuticle surface named as cuticular transpiration has been investigated extensively in the last few decades (Schönherr, 1976; Bargel et al., 2004; Samuels et al., 2008; Bernard and Joubès, 2013; Yeats and Rose, 2013). Theoretically, transport of compounds in the cuticle has two distinct routes where two types of compounds cross it (Schönherr, 2006). The polar path refers to polar aqueous pores where the cuticle is penetrated by water and polar compounds (Fernández and Eichert, 2009). However, only small molecular compounds may traverse these polar pores. The other route is the lipophilic one making use of the hydrophobic amorphous wax and cutin phase where lipophilic compounds easily pass through (Schreiber, 2005). Nevertheless, water can use both paths (Schönherr, 2000; Schreiber et al., 2001; Schreiber, 2005, 2006). So we can conclude that water transpires probably via polar path and lipophilic route *in vitro*. Unlike the low humidity conditions *ex vitro* the lower vapour pressure deficit in culture vessel facilitates water diffusion. When ambient air is highly filled with vapour, the cutin polymer swells, polysaccharides in the cuticle hydrates, the aqueous polar pores open up and are filled with water, additional polar routes are formed, and as a consequence, water flow accelerates in the cuticle (Kerstiens, 2006). That is why water permeability in the cuticle is enhanced by a factor of two to three in high humidity conditions (Schönherr, 2001) in tissue culture.

The increased cuticular transpiration might be beneficial for water pressure of the sap moving in phloem and therefore organic compound movement in plantlets *in vitro*. Higher transpiration and water permeability stimulate sugar movement upwards and subsequently more elements accumulation in the leaves as the sink region *in vitro*. Even so it is important to note not all increases in cuticular transpiration routinely or inevitably lead to elevated biomass. It was also reported cuticular wax mutant had strongly retarded growth in *dso* where DSO shows the highest identity and closer homolog of CER5 (Panikashvili et al., 2007). A possible explanation could be that synthesis of the long-chain fatty acids still maintains intact but function has been impaired of excretion and deposition in the cuticle (Pighin et al., 2004), the accumulation of these lipid compounds inside the cell influenced growth negatively. More efforts are required to investigate both cutin and wax synthesis and formation, cuticle structure and composition, and related influence of former factors on solution permeability and element translocation in tissue culture.

Stomata traits of *spch* and *epf1epf2 in vitro*

Stomata pores act as valves mainly mediating gas exchange in photosynthesis and water transpiration processes. Stomata are surrounded by pairs of guard cells and respond by closing in darkness but open when they sense blue light. Plant breeders could aim to enhance crop production through manipulation of stomatal density and stomatal opening (Jones, 1987). It is suggested that enhanced stomatal density could result in plants having higher stomatal conductance and gas exchange (Schlüter et al., 2003). As for stomatal functionality *in vitro*, ambiguous conclusions and inconsistent reports have been published. For example, stomata number of red raspberry *in vitro* was reported to be increased (Donnelly and Vidaver, 1984), however, stomata density was reduced in plum (Brainerd et al., 1981), stomata of *Solanum tuberosum* were functional to keep CO_2 at a

constant level in a closed vessel (Cournac et al., 1991), and stomata were incapable to close in *Prunus cerasus* (Marin et al., 1988).

Plants usually respond to environmental conditions regulating transpiration and CO_2 assimilation through stomata adaptation, e.g. by modulating stomatal density in the long term, and by stomatal opening and closing in the short term (Schlüter et al., 2003). EPF1 and EPF2 are involved in stomata development by negatively controlling asymmetric cell divisions in the stomata cell lineage (Hara et al., 2007; Hunt and Gray, 2009). The double mutant *epf1epf2* displays additive phenotypes with almost twice the stomatal density of the wild type, together with a somewhat smaller stomata size, and overall with increased transpiration (Hunt and Gray, 2009; Dow et al., 2014; Franks et al., 2015; Hepworth et al., 2015). SPCH is required for asymmetric division in the epidermis that produce stomatal guard cells, and *spch* and two paralogues mutants had almost no stomata or arrested stomata (MacAlister et al., 2007; Lampard et al., 2008). *In vitro*, the mutant lines *epf1epf2* and *spch* showed indeed an increased and decreased number of stomata, respectively, in comparison to wild type Col-0 (Figure 5).

When exploring relationships in stomatal density and stomatal opening size, reduced density often comes with increased size, and vice versa under various water supply levels or under varying CO_2 availability (Gindel, 1969; Uprety et al., 2002). It has been observed that an increase in stomatal density is accompanied with a decrease in stomatal size (Elias, 1995; Fraser et al., 2008). This inverse correlation between stomatal size and stomatal density might be related to EPF expression (Doheny-Adams et al., 2012). However, earlier reports also demonstrated that inverse correlations between stomatal density and stomatal aperture size do not seem to be consistent across and within species (Hetherington and Woodward, 2003; Zarinkamar, 2007; Lawson and Blatt, 2014). In this regards more controversial results were reported among species and across growth conditions (Franks et al., 2009; Silva et al., 2009; Yan et al., 2012). We found a larger number of stomata in *epf1epf2* and a smaller number of stomata in *spch* compared to the wild-type but only in *epf1epf2* the stomatal aperture size was negatively influenced in a significant way. Regardless of the shape or number of epidermal cells on the leaf surface, the presence and number of stomata and the stomatal size are the main surface feature determining leaf gas-exchange levels. The correlation between stomatal density and stomatal size is of physiological significance for plant carbohydrate production, on maximum stomatal gas-exchange capability.

Stomatal transpiration and growth *in vitro*

The stomatal number and opening determine maximum leaf conductance. Here, we observed that the *epf1epf2* mutant having a significantly higher number of stomata, albeit with a reduced aperture size, showed more water loss as an indicator for transpiration than the wild type control and the *spch* mutant which had fewer stomata. The level of stomatal transpiration was related to growth as demonstrated by fresh weight and dry weight measurements of the two mutants and the Col-0 control. Here we suggest that increasing transpiration *in vitro* might be a promising tool for improving growth characteristic *in vitro* through modulation of stomatal properties regardless of inverse relationship between stomatal size and its density. Doheny-Adams et al (Doheny-Adams et al., 2012) showed in Arabidopsis *in vivo* that plants with increased stomatal density transpired more than those with less stomata under controlled CO_2 conditions. In this research we showed that also under *in vitro* conditions a greater stomata number led

to increased water loss and biomass formation, suggesting a link between transpiration and growth *in vitro*.

Nutrient assimilation and the subsequent transport to the sink regions are correlated with water uptake both *in vivo* and *in vitro*. To our knowledge, research is quite scant in the field of correlation between stomata traits, water transpiration and nutrient movement *in vitro*. Plants acquire dissolved nutrients through liquid flow driven by transpiration (Barber, 1962). It is hypothesized here that under *in vitro* conditions also sucrose, which is abundantly present in the medium inside the containers, moves upwards in plantlets by a flow driven by transpiration. We demonstrated the presence of both stomatal as well as cuticular transpiration under *in vitro* conditions. It seemed cuticular transpiration led to a higher biomass accumulation than stomatal transpiration did (Figure 7) with the mutants used in this study. However, we could not quantify in a direct comparison the transpiration levels and the contributions made by the two transpiration routes in one system. Our hypothesis still might hold true that transpiration contributes to plantlet growth *in vitro* via cuticle route and stomata pathway. But there could be more factors which play a role in growth *in vitro* of these mutants. While *att1* and *epf1epf2* have a comparable level of transpiration there exists a significant difference in growth between the two, again suggesting that in addition to transpiration something else might be vital for growth. Another possibly relevant observation was that the *att1* mutant had a greater extended lateral root area and primary root area in the medium (data not given). This increase in root system size could have contributed to the observed increase in growth in this mutant. Root architecture and size influences water and nutrient uptake, and, hence, plant growth and yield (Gilroy and Jones, 2000; Šimůnek and Hopmans, 2009; Chapman et al., 2012).

Nevertheless both stomatal transpiration and cuticular transpiration contribute to the induction of a liquid flow carrying nutrients leading to biomass accumulation *in vitro* and the role of cuticular transpiration *in vitro* should not be neglected. We suggested earlier that restrictions in nutrient assimilation *in vitro* might be lifted through optimizing transpiration. A possible way to do this is by adding S-carvone, which is applied exogenously to plant tissues preventing the synthesis of wound related deposition of lipid–phenolic complexes (e.g. suberin) (Oosterhaven et al., 1995), leading to a delayed decline in hydraulic conductance and increased solution uptake and higher fresh weight *ex vitro* (He et al., 2006; Damunupola et al., 2010). Another option could be by applying the fungal phytotoxin fusicoccin, a plasma membrane H^+-ATPase activator which widely opens stomatal apertures (Akita et al., 2018) and leads to enhanced growth *ex vitro* (Siemieniuk and Karcz, 2015).

Conclusion

The purpose of this research was to demonstrate the physiological role of stomata and cuticle in their contribution to transpiration and nutrient transport, and therefore determining biomass accumulation or growth in *in vitro* micropropagated plants. Through molecular and physiological analysis it was found that the *att1* cuticle mutant line had increased permeability, transpiration and growth *in vitro*, and that stomata mutant *epf1epf2* had increased transpiration and biomass production *in vitro* while the wax mutant line *cer5* and the no-stomata mutant line *spch* not. Taken together, this study provided new insights that growth of micropropagated plantlets *in vitro* could be enhanced by optimization of transpiration conditions through changing internal culture

vessel humidity as well as plant properties via regulating of stomata and cuticle characteristics. We provide evidence for the correlation between stomata conductance and cuticle transpiration, and biomass build-up *in vitro*. More research is required for exploring stomatal transpiration, cuticle transpiration and nutrient transportation in other crops to establish the general character of our observations and hypothesis.

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Supplemental table list of primers used in the Q-RT-PCR analysis

Gene target	Primer sequence (in 5'-- 3' order)	Accession no.
Cer5-F	TGTCTCGCATTGGGCTTTCT	
Cer5-R	ACCTTTGGCAGATCGGGAAG	AT1G51500
Att1-F	CGACCGGTTGGTTTACCTGA	
Att1-R	CGAATTTTCCATCGTCCGGC	AT4G00360
Epf1-F	CATGTAGGAATGACCGGGGC	
Epf1-R	CGACGGATGCACACACAAAG	AT2G20875
Epf2-F	AAAAACACGGTCAATGGCGG	
Epf2-R	GGCGACTGAGCATTCGAAAC	AT1G34245
Spch-F	CGTGGAACGTAACCGGAGAA	
Spch-R	TCTTGGGCTTAGAACAGGCG	AT5G53210
Actin2-F	TCTTAACCCAAAGGCCAACA	
Actin2-R	CAGAATCCAGCACAATACCG	At3G18780

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CHAPTER 3

SWEET11 and SWEET12 genes mediate exogenous sucrose influx and translocation in tissue culture

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Abstract

Micropropagated plantlets are usually grown *in vitro* on artificial media supplemented with sucrose. This exogenous sucrose plays a critical role in the plantlet's growth and development although photosynthesized sucrose also matters. The roots of *in vitro* plantlets are able to take up sucrose from solidified artificial medium. However, the process of sucrose translocation, e.g. via the apoplast or via transmembrane migration, in roots *in vitro* has not been studied in great detail yet and neither has the role of sucrose transporter genes and the proteins they encode. For this, we looked at gene expression levels of seventeen SWEET and nine SUC genes in leaves and roots from *in vitro* as well as from *ex vitro* plants. The gene expression studies showed that expression differed between leaves and roots and between *ex vitro* and *in vitro* plants. Looking at *in vitro* roots the upregulated gene expression of SWEET11 and SWEET12 were among the most prominent differences. For continuation, we included mutants, in particular mutants in *sweet11* and *sweet12* as single mutants, and one double mutant *sweet11;12* and transgenic lines in which the promoters of SWEET11 and SWEET12 were combined with the GUS reporter gene. It was observed that the single mutants *sweet11* and *sweet12* did not exhibit a noticeable morphological change under *in vitro* conditions whereas the double mutant *sweet11;12* did display reduced growth *in vitro,* hence we focused on the double mutant. The GUS reporter analysis revealed that SWEET11 and SWEET12 promoters were induced by sucrose available in the media and both were expressed in the vasculature. Next, we determined carbohydrate levels and studied growth measuring fresh weight and dry weight of plants as well as of isolated root cultures. Increasing concentrations of exogenous sucrose induced a reduction in primary root length of *in vitro* seedlings in wild-type Col-0, while seedlings of the double mutant displayed roots with a less reduced primary root length at increasing sucrose concentrations compared to Col-0, and showed a higher amount of sucrose in roots. Isolated root cultures showed that in the double mutant *sweet11;12* sugar uptake in the roots might be lower, but not zero. Despite the lower uptake in the isolated roots of the double mutant, the sucrose still accumulated in the roots in intact plants. Sucrose uptake can be visualized using the fluorescent sucrose analogue, esculin. We investigated whether sucrose translocation in the *sweet11:12* double mutant would be impaired by monitoring the uptake of esculin in protoplasts but no significant differences were found. These results taken together imply that SWEET11 and SWEET12 are involved in mediating sucrose uptake from the medium and translocation within the root to the phloem, possibly via the transmembrane route and subsequent translocation upwards in *in vitro* grown *Arabidopsis* seedlings. Conditions stimulating sucrose translocation *in vitro* might help in increasing propagation efficiency and plant quality.

Keywords: *Arabidopsis thaliana*, SWEET11, SWEET12, sucrose uptake and translocation, tissue culture

Introduction

To maintain sustainable growth and development autotrophic plants in *ex vitro* rely primarily on photosynthesized soluble sugars as key signalling molecules, energy source and building blocks. Membrane sugar transporter proteins play pivotal roles in mediating sucrose transport within plants on a cellular level. The sugar transporter genes are involved in the transport process of sugars from leaves, as the place of biosynthesis (source), to roots or seeds, as the site of utilization (sink). On a cellular level sugars are suggested to migrate through plasmodesmata, as narrow cylindrical ducts which connect the symplasts of neighbouring cells, in the so-called symplastic pathway (Lucas et al., 1993) while at some tissue interfaces sugars are thought to move via an apoplastic pathway (Giaquinta, 1983). In the apoplastic route, one transporter mediates sugar efflux from a producer cell into the apoplast in a secreting process whereas another transporter regulates re-influx from the apoplast into the cell cytoplasm (Lalonde et al., 2004), therefore sucrose transporters play a key role in the apoplastic route. Sucrose is the main form of the photoassimilate in apoplastic translocation. The photoassimilate is transported from mesophyll cells, where solar energy is used to convert CO_2 into organic carbon, into phloem parenchyma cells, entering the sieve element–companion cell complex (SE/CC)(Yadav et al., 2015). Within the phloem loading process SWEET transporters (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS) and SUC/proton symporters (Sucrose transporters/Sugar transporters) mediate apoplastic uploading and unloading (Carpaneto et al., 2005; Le Hir et al., 2015). SWEET genes regulate sucrose export from the phloem parenchyma cells symplast into the apoplast whereas SUC/proton genes mediate sucrose reloading from the apoplast into the companion cell/sieve element complex driven by proton motive force (Riesmeier et al., 1992; Sauer, 2007; Chen et al., 2012). Long distance sucrose transport in a bulk flow is driven by osmosis and phloem hydrostatic pressure in sieve tubes which are formed by connecting sieve elements end to end (Mnch, 1930; Lalonde et al., 2003; Patrick, 2013). After arriving at its destination sugar is unloaded from the phloem tube through the symplast by plasmodesmata or through a transmembrane-apoplast process (Yadav et al., 2015). Additionally, sugar can be taken up by sucrose importer or hexose importer proteins. Sucrose might be hydrolysed into glucose and fructose by cell wall invertases (Eom et al., 2015). The hexoses resulting from sucrose hydrolysis can be taken back up by SUCs in the extracellular space in vegetative sinks (Carpaneto et al., 2005; Braun and Slewinski, 2009; Kühn and Grof, 2010) as well as by monosaccharide transporters (Pego and Smeekens, 2000; Sherson et al., 2003).

The Arabidopsis SWEET sugar transporters are identified by co-expression in human HEK293T cells which do not have significant sucrose uptake activity but have low glucose uptake capability (Takanaga et al., 2008; Chen et al., 2010; Frommer et al., 2013). Sucrose transporters are analysed using FRET sucrose sensors in HEK293T cells (Chen et al., 2010; Chen et al., 2012; Lin et al., 2014). According to these investigations SWEETs are hepta-helical proteins composed of a repetition of three transmembrane domains connected by linker-inversion (Chen et al., 2010). To make a phylogenetic tree of SWEETs only proteins containing two triple-helix-bundle repeats are filtered, and thus phylogenetic relationships are established, that is, clade I (SWEETs 1–3), clade II (SWEETs 4–8), clade III (SWEETs 9–15), and clade IV (SWEETs 16–17)(Chen et al., 2010; Eom et al., 2015). The number of SWEETs varies in species and the membership of SWEETs in a clade does not provide reference for predicting its physiological role (Eom et al., 2015). As for function analysis, SWEETs are thought to play roles in nurturing the

gametophyte (Bock et al., 2006), male fertility (Guan et al., 2008), host susceptibility factor (Chen et al., 2010) and so on. SWEET9 functions as a sucrose transporter and mediates nectar production (Ge et al., 2000; Lin et al., 2014). SWEET11 and SWEET12 mediate sucrose translocation out of leaves (Chen et al., 2012). SWEET15 is involved in hexose remobilization and senescence (Seo et al., 2011; Zhou et al., 2014). SWEET11, SWEET12 and SWEET15 might regulate seed and embryo development (Chen et al., 2015b; Sosso et al., 2015).

Micropropagated plantlets growing on artificial media (either solid or liquid) are cultured for many purposes, such as miniature tubers formation of potato and sweet potato (Hussey and Stacey, 1981; Ng, 1988) and protocorms propagation of orchids (Tanaka et al., 1975; Chen et al., 2002) or gladiolus (Ziv et al., 1970; Ziv, 1989). The growth success in tissue culture depends on the composition of the growth medium; and on a few physical parameters, such as light and temperature; and on the efficiency of the nutrient uptake, translocation and utilization. To satisfy the requirements for growth, plant tissue culture media are often composed of inorganic ions, vitamins, plant growth regulators (PGRs) and sucrose as carbohydrate source (George et al., 2008a; Hand and Reed, 2014; Deepthi and Satheeshkumar, 2017). The uptake of all these elements, however, is generally influenced by different factors such as medium type, gelling agent, concentration, pH, temperature and explant physiological status (Williams, 1993; Scholten and Pierik, 1998; Ramage and Williams, 2003; George et al., 2008a, b). Nutrient flow, in particular sucrose translocation *in vitro,* should be of importance for plantlet growth and multiplication but has not been a priority of studies in plant tissue culture up till now.

Plants *in vitro* are thought to have insufficient photosynthetic competence (Van Huylenbroeck and Debergh, 1996), and have a lower accessibility to CO_2 inside the vessel (Hazarika, 2003). This is why tissue culture conditions are often semi-autotrophic (Hazarika, 2003); the addition of sucrose to the culture media is required to meet the carbon source for the plantlet's multiplication and growth (Marino et al., 1993; Hazarika, 2003). The carbohydrate nutrition is required in tomato roots: external sucrose increases growth and anatomy of excised roots (Street and McGregor, 1952). The exogenous sugar supply also increases plantlet starch reserves in micropropagation and favours plantlet future adaptation and acclimatization during transfer to *ex vitro* conditions (Pospóšilová et al., 1999). Nevertheless, there are also many reports on the negative correlation between sugar addition and physiological processes such as photosynthesis (Hdider and Desjardins, 1994; Serret et al., 1996) and sugar enzymes activity involved in carbon assimilation (Kilb et al., 1996). As almost no research has been reported on the molecular mechanism of exogenous sucrose transport *in vitro*, it is still not clear how exogenously supplied sucrose is translocated and what sucrose transporters are involved in regulating sucrose translocation from the medium (source) to the plants' parts (sink). The priority in this research is to understand sucrose translocation starting from roots *in vitro*.

The research presented here attempted to provide answers to questions on whether and which sucrose carrier genes are involved in the assimilation and translocation of exogenously supplied sucrose, and what their contribution is to plant growth *in vitro*. First, we studied the expression of 17 SWEET genes and 9 SUC genes both in leaves and in roots and under both *in vitro* and *ex vitro* conditions. Later, we focused on two of them, SWEET11 and SWEET12, which genes are known to be linked to sucrose translocation,

and studied their contribution to the uptake and translocation of sucrose in roots *in vitro*. Using single and mainly double mutants we investigated the roles that SWEET11 and 12 play in plant growth, in determining root length, and in sucrose content. The effect of sucrose on SWEET11 and SWEET12 gene expression was analysed as well as the localization of SWEET11 and SWEET12 in roots. In addition, the growth of excised roots of the double mutant *sweet11;12* was examined to investigate the role of sucrose uptake in roots in isolated root cultures. To understand the role of SWEET11 and SWEET12 in sucrose translocation across membranes, an esculin uptake assay was performed on mesophyll protoplasts and root protoplasts. Our research provides the start of research aimed at better understanding the exogenous sucrose translocation *in vitro*.

Materials and methods

Plant materials and growth conditions

All Arabidopsis transgenic lines were in the Columbia-0 background and homozygous (Chen et al., 2012), and obtained from the Arabidopsis Biological Resource Centre (ABRC): *p*AtSWEET11:AtSWEET11-GUS, *p*AtSWEET12:AtSWEET12-GUS, *sweet11* T-DNA insertion mutant line (SALK_073269), *sweet12* T-DNA mutant line (SALK_031696), the *sweet11;12* double mutant line (CS68845) was made by crossing *sweet11* and *sweet12*. For *ex vitro* purposes, plants were grown in small pots filled with a vermiculite and solarite soil mix (ratio 1:2)(Kekkilä Oy, Äyritie, Finland) under 16 hours light, 8 hours darkness, 21°C in day time, 19°C at night, and at a relative humidity of 70%. For analysis of *in vitro* growth, *Arabidopsis thaliana* seeds were surface sterilized with 70% (v/v) ethanol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for one minute and 2% (w/v) sodium hypochlorite (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 15 minutes. Later, the seeds were rinsed 3 times in sterilized water and then these seeds were placed on Petri dishes containing full strength MS including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands), 1% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin-agar (Duchefa, Haarlem, The Netherlands)(pH 5.7-5.8). The seeds were stratified in the dark at 4°C for 3 days and afterwards they were transferred to 22°C under conditions of 16 hours light, 8 hours darkness and 50 µmol m^{-2} s^{-1} illumination (Philips Master TL-D 36)(Philips, Poland) in a climate chamber (Conviron BDR16)(Conviron Germany GmbH, Berlin, Germany). The Arabidopsis seedlings were grown in standard Petri dishes or in square Petri dishes (120 × 120 × 17mm)(Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) with full strength MS medium including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands)(pH 5.7-5.8), and 1% (w/v) Daishin-agar (Duchefa, Haarlem, The Netherlands) supplemented with 0, 2%, 4% and 6% (w/v) sucrose separately.

Quantification of gene expression in leaves and roots by RT-PCR analysis

All RNA was isolated from 3-weeks-old *Arabidopsis thaliana* leaves and roots grown in two conditions. *Ex vitro* Arabidopsis were grown on small pots filled with vermiculite and solarite soil mix (ratio 1:2)(Kekkilä Oy, Äyritie, Finland) and *in vitro* Arabidopsis were grown on Petri dishes containing full strength MS including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands), 1% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin-agar (Duchefa, Haarlem, The Netherlands). RNA was isolated from roots and leaves by using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Next, cDNA was obtained by using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions: the 20 µL reaction mixture containing 11 µL DNase treated RNA, 4 µL 5X iScript reaction buffer, 4 µL Nuclease-free water, 1 µL iScript reverse transcriptase, was incubated in a program of 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, 4°C for 5 min, 85°C for 5 min, and subsequently held at 10°C until further use. The resulting cDNA was diluted and used as the template for expression quantification. Gene specific primer pairs are given in the supplementary table and were validated prior to use. The primer sequences were modified from Dubey et al. (2013) and Guo et al. (2014). The amplification was performed using a SYBR Green kit (Bio-Rad Laboratories, Hercules, USA) on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's instructions. The real-time PCR assays were performed as follows: 95°C

for 10 minutes, 1 cycle; 40 cycles of 95˚C 15 seconds, 55˚C for 1 minute; 95˚C for 10 minutes; 65˚C for 5 seconds and increased to 95˚C by 0.5˚C. The relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ with UBQ-10 serving as the reference gene (Czechowski et al., 2005) where C_t represented the threshold cycle (Livak and Schmittgen, 2001).

Mutant screening assay

After 21 days of growth Arabidopsis mutant lines were screened for expression using RT-PCR. Total RNA was extracted from leaves of various mutant lines using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA was subjected to removal of genomic DNA using RNase-free DNase I (Qiagen, Hilden, Germany). Single strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Expression levels were tested by real-time PCR using primers shown in the supplementary table. All real-time PCR assays were performed as follows: 95˚C preheating for 10 min, 1 cycle; 40 cycles of 95˚C 15 sec, 55˚C 30 sec; 95˚C for 10 min; 65˚C to 95˚C by 0.5˚C increment for 5 sec. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using Actin2 as the reference gene.

Growth measurement

Arabidopsis plants growing in Petri dishes under the appropriate experimental conditions were collected for weight analysis. The plants were carefully removed from the medium, soaked in water to dispose of medium adhering to them, and rubbed dry in tissue paper. These plants were subsequently placed in empty tubes of which the weight was already known and were weighed by a 0.01 mg resolution balance, XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands). Subsequently, the plants were dried in an oven (Omnilabo International B.V., Breda, The Netherlands) at 70 °C overnight and were then weighed using the 0.01 mg resolution analytical balance again.

Sugar extraction and determination

At the end of the light cycle, leaves and roots were collected and excised from at least 30 different 4-weeks-old plants per mutant line. The tissues were freeze dried (Freeze Dryer)(ilShin Biobase Europe B.V., Ede, The Netherlands) overnight, and subsequently weighed (XPE105 Analytical Balance)(Mettler Toledo B.V., Tiel, The Netherlands) and used for carbohydrate extraction. Freeze-dried material, 20 mg, was incubated in a 12 mL glass tube with 1 ml 80% (v/v) ethanol for extraction three times. The extraction was done at 80 °C in a thermoshaker (Salmenkipp, Breukelen, The Netherlands) at a speed of 500 rpm for 40 minutes each time. After that, the samples were centrifuged at 4000 rpm for 10 minutes using Heraeus Multifuge 3L-R (Kenodo Laboratory Products, Asheville, USA), and then the supernatant was transferred to a new tube by a glass Wilhelm Ulbrich Pasteur pipette (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). The supernatant was dried in a vacuum using the RapidVap (Beun De Ronde, Abcoude, The Netherlands). The residues were resuspended and dissolved in MQ water. The extracted small-molecule soluble sugars were then tested in High Performance Liquid Chromatography (HPLC) as described by Schneider et al. (2008). The soluble sugars were separated by HPLC with a Dionex column (Dionex ICS 5000)(Thermo Fisher Scientific B.V., Waltham, MA USA) while the eluent was 500 mM NaOH and the process took 80 minutes. The chromatograms were analysed using the Chromeleon program

(Dionex)(Thermo Fisher Scientific B.V., Waltham, MA USA) while the known sucrose standard samples were used to identify peaks in an electrochemical detector (Dionex)(Thermo Fisher Scientific B.V., Waltham, MA USA). The quantitative calculation was performed using Chromeleon software (Dionex)(Thermo Fisher Scientific B.V., Waltham, MA USA) based on calibration curves.

GUS histochemistry

Germinated seeds were transferred from medium lacking sucrose to media supplemented with varying concentrations of sucrose (0, 2%, 4% or 6%) and were subsequently grown for three-to-four weeks. After this, the plants were transferred to 20 ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) histochemical reagent in a 50 ml blue cap tube (Duchefa, Haarlem, The Netherlands) according to Vitha et al. (1995). To get the X-Gluc reagent 1.0 ml dimethyl formamide containing 0.0118 mmol X-Gluc was dissolved in 10 ml 50 mM phosphate buffer (pH 7.2). A 200 mM phosphate buffer stock (pH 7.2) was prepared by mixing 39 ml 200 mM NaH_2PO_4 and 61 ml 200 mM Na_2HP0_4. Plants were firstly incubated in X-Gluc in a stove (Omnilabo International B.V., Breda, The Netherlands) at 37 ^oC overnight, after that plants were rinsed in 70% (v/v) ethanol to remove excessive chlorophyll for further observation. The roots were observed and photographed using a stereomicroscope, a Zeiss Stereo Discovery V8 Zoom Stereo Microscope (Carl Zeiss Microscopy, White Plains, United States). For staining cross-sections of roots, the roots were stained in X-Gluc reagent without pre-fixation and after staining the roots were fixated and embedded in plastic. Lateral and primary roots were fixed in 5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight, washed in 0.1 M phosphate buffer (pH 7.2) for 15 minutes at least 4 times and in water 2 times, dehydrated in increasing concentrations of ethanol (10%, 30%, 50%, 70%, 96% and 100%)(v/v) for 20 minutes per step, infiltrated by using Technovit Glycol Methacrylate Kit 7100 (Kulzer Technique, Wehrheim, Germany), and finally embedded in moulds. All samples were cut in 9 μm transverse sections utilizing a Reichert-Jung ultra-cut ultramicrotome (Leica Reichert Jung, Bayreuth, Germany). The sections were observed and recorded on glass slides using a Zeiss Axiophot light microscope (Carl Zeiss Microscopy, White Plains, United States) equipped with AxioCam ERc5S digital camera (Carl Zeiss Microscopy, White Plains, United States).

Root phenotype assay

Selected *Arabidopsis thaliana* seedlings of similar size were transferred to vertical square Petri dishes (120mm × 120mm × 17mm)(Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) which contained 0, 2% or 4% (w/v) sucrose respectively, full strength MS including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin-agar (Duchefa, Haarlem, The Netherlands)(pH 5.7-5.8). The plants growth was under a photoperiodic cycle of 16 hours light and 8 hours darkness at 22°C and a light intensity of 50 μmol m^{-2} s^{-1} (Philips Master TL-D 36)(Philips, Poland). After 2 weeks of culture, photographs (Canon EOS 4000D)(Canon Inc., Tokyo, Japan) were taken together with micrometre ruler as background and main root lengths were measured using Image-J (United States National Institutes of Health and the Laboratory for Optical and Computational Instrumentation in University of Wisconsin, USA). Around 15 seedlings were tested in each treatment.

Measurement of root activity in liquid root culture

2-Weeks-old cultured Arabidopsis were collected and their primary roots were excised and cut into fragments. The same number of 1-cm-length root fragments from around 10 plants were incubated in one 125 ml Erlenmeyer flask (Duchefa, Haarlem, The Netherlands) containing 30 ml root culture medium (ARC)(pH 5.7) comprised of full strength MS including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands), 1% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 0.285 μM indole-3-acetic acid (IAA)(Duchefa, Haarlem, The Netherlands)(Czakó et al., 1993). All the flasks, 6 flasks per line, were covered by aluminum foil to create dark conditions and were placed in a shaker at approximately 50 rpm (IKA-Labortechnik AS 501.4, Staufen, Germany) in a climate chamber at 21°C. In the flasks extensive adventitious roots were regenerated from excised fragments in 2 weeks and they were used for further measurements. The fresh weight of these adventitious roots was measured (XPE105 Analytical Balance)(Mettler Toledo B.V., Tiel, The Netherlands); the medium pH change in flasks (pH probe)(Mettler Toledo B.V., Tiel, The Netherlands) was determined; and the root phenotype was photographed (Axiophot light microscope)(Carl Zeiss Microscopy, White Plains, United States).

Protoplast esculin influx assay

Protoplasts were generated from Arabidopsis leaves as described by Yoo et al. (2007). The 10-20 selected well-expanded healthy leaves with a similar size excised from 4-weeks-old Arabidopsis which were grown in a rectangular container (107 × 94 × 96 mm)(Duchefa, Haarlem, The Netherlands), were chopped into 0.5 mm leaf strips using sharp razors. Protoplasts were released by submerging these leaf strips in 20ml fresh enzyme solution 4 hours for digestion and being diluted with 20 ml W5 solution next to being filtrated through cheese cloth. The fresh enzyme solution was composed of 20 mM MES (pH 5.8)(Duchefa, Haarlem, The Netherlands), 1% (w/v) cellulase Onozuka R10 (Duchefa, Haarlem, The Netherlands), 1% (w/v) macerozyme Onozuka R10 (Duchefa, Haarlem, The Netherlands), 0.5 M sucrose (Duchefa, Haarlem, The Netherlands), 10 mM $CaCl_2$ (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 20 mM KCl (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.1% (w/v) albumin bovine serum (BSA)(Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and was filter sterilized (Zhai et al., 2009). The W5 solution consisted of 0.1% (w/v) glucose (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 5 mM KCl (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 154 mM NaCl (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 125 mM $CaCl_2$ (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 2 mM MES-KOH (pH 5.8)(Duchefa, Haarlem, The Netherlands)(Zhai et al., 2009). Isolated protoplasts were counted under a light microscope using a haemocytometer and after that the protoplasts were suspended at 2×10^5 ml^{-1} in W5 solution supplemented with 2 mM esculin (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) at pH 5.6. The protoplasts were incubated for half hour in a shaker (IKA-Labortechnik AS 501.4, Staufen, Germany) at approximately 20 rpm in a climate chamber at 21°C for uptake and subsequently these protoplasts were washed 3 times, and transferred to new W5 solution without esculin. During this procedure the protoplasts were kept in the dark by covering the tubes with aluminium foil. The protoplasts were then observed on glass slides using Axiophot light microscope (Carl Zeiss Microscopy, White Plains, United States) equipped with AxioCam ERc5S digital camera (Carl Zeiss Microscopy, White Plains, United States). A solid state diode was used to provide

excitation; light emission in wavelength between 410 nm and 450 nm was selected by a green filter. Exposure time was set at 4000 s for fluorescence analysis. Images were obtained in bright field, fluorescent field, and merged field and analysed using Image-J. The fluorescence of each protoplast was further measured using image J-win-java8 (United States National Institutes of Health and the Laboratory for Optical and Computational Instrumentation in University of Wisconsin, USA). There was measurable uptake of esculin in Col-0 protoplasts (but not all protoplasts) indicating this approach could be used as indirect measure of sugar uptake. Apart from mesophyll protoplast isolation root protoplasts were generated in a similar procedure from *in vitro* grown Arabidopsis as described by Demidchik et al. (Demidchik et al., 2002; Demidchik and Tester, 2002; Demidchik et al., 2004) and were analysed for esculin uptake.

Statistical analysis

All data were subjected to a one-way ANOVA together with Bonferroni-Holm adjustment test or t test using statistical package GraphPad Prism (GraphPad Software, San Diego, USA) or excel. Values were displayed as means ± SEM. Gene expression was analysed by using a statistical computing R language package for expression studies (R Development Core Team, University of Auckland, New Zealand). All data were obtained from at least two or three independent replications. Asterisks above each bar represent statistically significant differences (*, $P<0.05$ and **, $P<0.01$). Different letters above each bar represent statistically significant differences ($P < 0.05$).

Results

Expression of sugar transporter genes in different tissues

The gene expression analysis showed that sucrose carrier genes (SUCs; n=9) had different expression patterns. The same was the case for the SWEET genes (n=17), known as hexose and sucrose transporters (Figure 1). The transcriptional differences were found between leaves and roots; and between the two conditions, being *ex vitro* and *in vitro*. Under *ex vitro* conditions SWEET11, SUC2 and SUC3 showed a higher expression level than SWEET1, which was taken as reference, in leaves whereas SUC3 and SUC4 dominated in roots. The *ex vitro* expression patterns deviated substantially from the *in vitro* situation where SWEET11, SWEET12, SWEET16, SWEET17, and SUC3, SUC4 showing relatively high expression in roots and SWEET11 and SWEET12 proved to be highly expressed in leaves next to SWEET16 and SWEET17. SUC3 and SUC4 were relatively low in expression in *in vitro* leaves, contrary to *in vitro* roots. It seemed that SWEET11 and SWEET12 expression in roots was linked to the *in vitro* condition with sucrose in the medium and therefore we attempted to understand their roles in the translocation of exogenously supplied sucrose and in supporting *in vitro* growth.

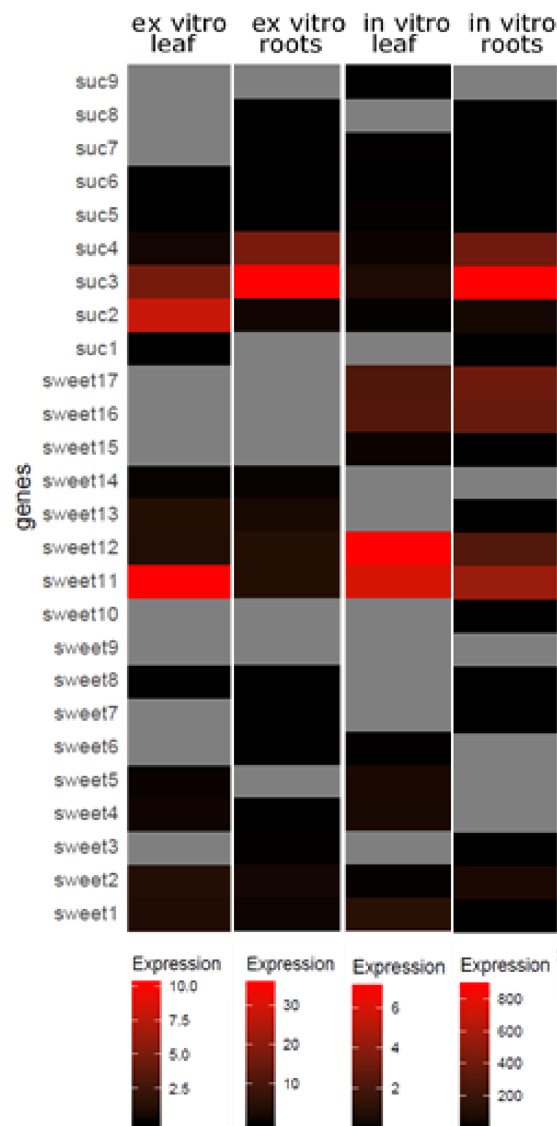


Figure 1. Expression patterns of SWEET and SUC genes in leaves and roots grown in both *ex vitro* and *in vitro* conditions. The leaves and roots used to isolate RNA originated from 2-weeks-old Arabidopsis plants. Expression profiles of sugar transporters in leaves *ex vitro*, roots *ex vitro*, leaves *in vitro*, roots *in vitro* are shown with the expression level of SWEET1 set at 1 in each column. Grey means no data available; black shows expression in low level to that of SWEET1; red indicates higher expression levels.

Characterization of the *sweet11*, *sweet12* single and *sweet11;12* double mutant lines

By quantitative PCR it was verified that SWEET11 and SWEET12 expression levels in both single and double mutant lines were very low. The T-DNA insertion mutant line *sweet11* (Figure 2A) had low expression for SWEET11 as did *sweet12* for SWEET12 (Figure 2B) and *sweet11;12* for both genes (Figure 2C) with the expression level in the wild-type Col-0 set at 1. The stable level of Actin2 (Figure 2D), used as internal control, and low levels of SWEET11 and SWEET12 (Figure 2E) are also shown as bands in gel electrophoresis.

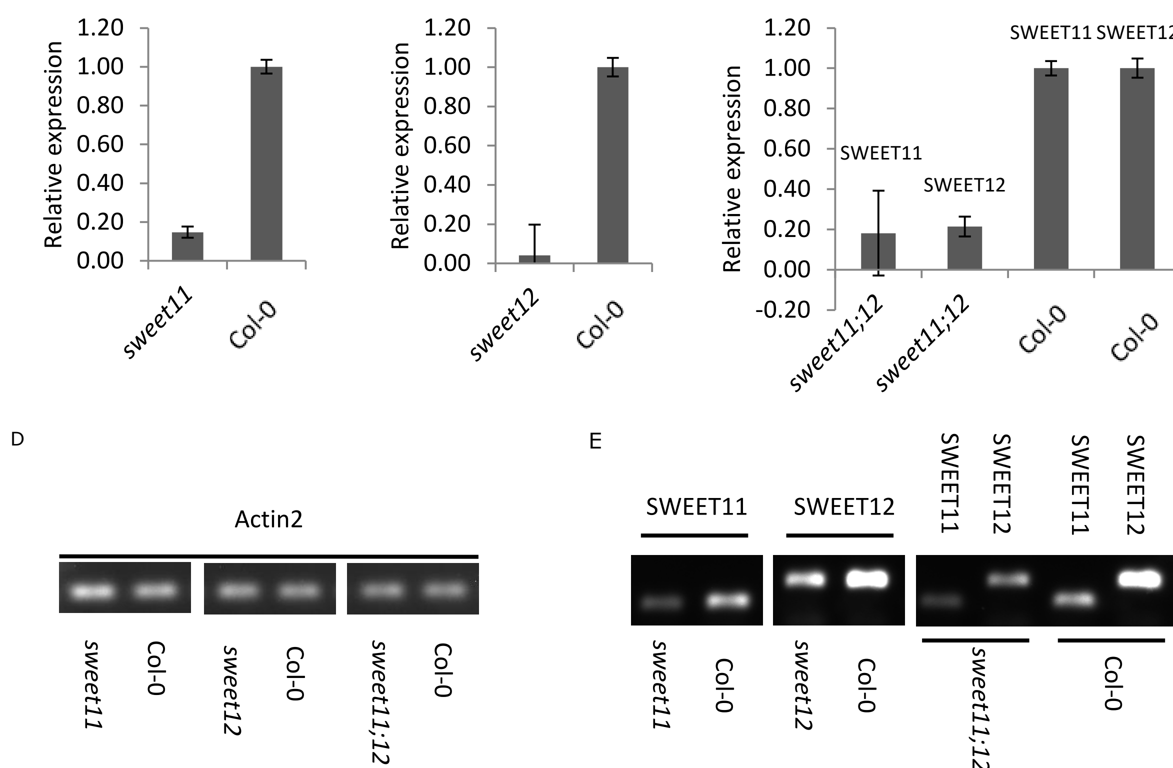
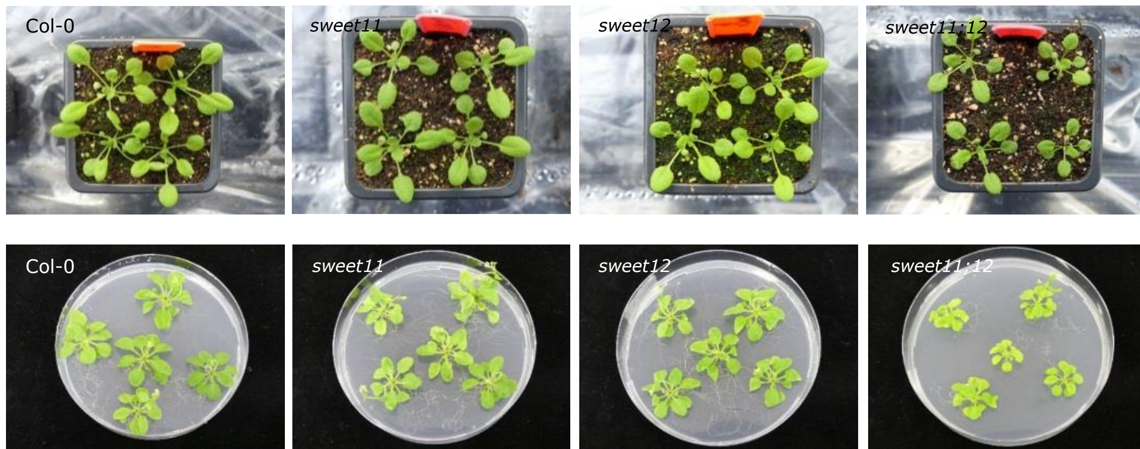


Figure 2. Expression profiles of the *sweet11, sweet12* and *sweet11;12* mutant lines. A, B, C, RT-PCR testing SWEET11 and SWEET12 gene expression levels in *sweet11* (A), *sweet12* (B), and *sweet11;12* (C) mutant lines relative to Actin2 with the expression level in Col-0 set at 1. D, Actin2 expression levels on gel in *sweet11, sweet12* and *sweet11;12* as internal control. E, the low transcript levels for SWEET11 and SWEET12 in *sweet11, sweet12* and *sweet11;12* mutant lines on gel after qPCR.

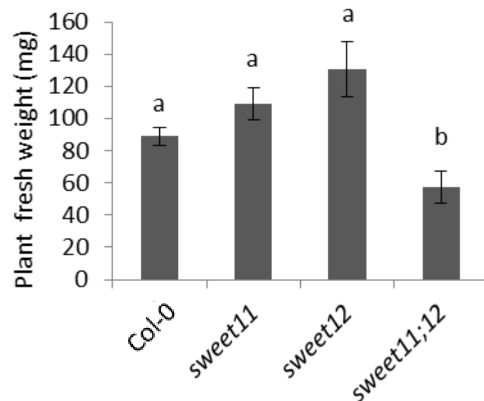
Growth profile of *sweet11*, and *sweet12* mutant lines *in vitro*

AtSWEET11 and AtSWEET12 are closely-related paralogs, which have a high identity in amino acid sequences, facilitating sucrose translocation across the membrane (Chen et al., 2012). It is interesting to investigate their influence on *in vitro* growth and on exogenous sucrose flux in plants grown *in vitro*. We examined the phenotypes and growth of the mutants *sweet11* and *sweet12* which might have reduced sucrose efflux. Compared to wild type Col-0 the mutant lines *sweet11* and *sweet12* did not exhibit any noticeable change in morphology or phenotype under *ex vitro* greenhouse growth conditions (Figure 3A, upper panel). The double mutant line *sweet11;12* was obviously smaller compared to wild-type Col-0. Similarly *in vitro*, the single mutants *sweet11* and *sweet12* failed to show altered growth phenotypes (Figure 3A, lower panel). Possibly, this is because clade III SWEETs, typically involved in cellular sucrose efflux processes (Chen et al., 2010; Lin et al., 2014), are genetically redundant. The double mutant plants *sweet11;12* displayed a reduction in size *in vitro* (Figure 3A, lower panel). Weight analysis as indicator for growth showed that the sample of *sweet11;12* line had 57.2 mg fresh weight and 2.02 mg dry weight, while wild-type Col-0 had 88.9 mg fresh weight and 5.31 mg dry weight after 4 weeks of growth *in vitro* (Figure 3B and 3C). Generally, both under *ex vitro* growth conditions and under tissue culture conditions, the seedlings of the double mutant showed a smaller plant size and lower weights than the wild type. To investigate the physiological function of SWEET11 and SWEET12 *in vitro*, the double mutant line *sweet11;12* was chosen for further analyses.

A



B



C

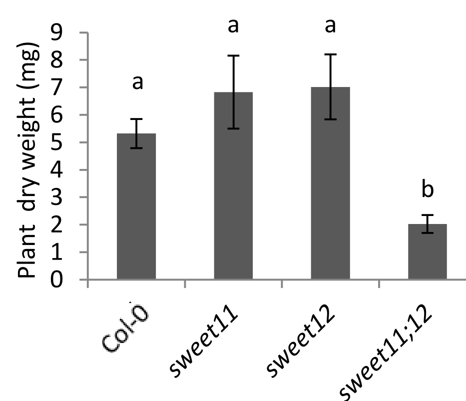


Figure 3. Growth characteristics of *sweet11* and *sweet12* single mutant lines and the *sweet11:12* double mutant line. A, the growth phenotypes of wild-type Col-0, *sweet11*, *sweet12*, and *sweet11;12 ex vitro* (upper) and *in vitro* (lower). B, the fresh weights of wild-type Col-0, *sweet11*, *sweet12*, and *sweet11;12* grown *in vitro.* C, the dry weights of wild-type Col-0, *sweet11*, *sweet12*, and *sweet11;12* grown *in vitro.* (mean ± SEM, n = 5 ~ 10 plants). Different letters above each bar represent statistically significant differences ($P < 0.05$).

The effect of sucrose on roots of *sweet11;12* plants grown *in vitro*

As it is assumed that *in vitro* SWEET11&12 regulate sucrose uptake, radial translocation in roots and the subsequent upward transport, we wanted to monitor root growth of the double mutant line *sweet11;12* and measure the accumulated soluble sugar content in roots. Our hypothesis was that the impaired translocation of exogenously supplied sugar in *sweet11;12* would lead to higher sugar accumulation in the roots, possibly affecting root growth and root length. On sugar-free media a reduction of primary root length was exhibited in *sweet11;12* plants, 3.82 cm compared to Col-0 with 4.7 cm. The primary root length of *sweet11;12* plants in the presence of sucrose displayed a size of 2.78 cm on media containing 2% sucrose and was further reduced at 4% sucrose to a size of 2.12 cm. The primary root length on media containing 2% and 4% sucrose in wild type Col-0 showed a similar decreasing trend with sizes of 2.96 cm and 1.95 cm respectively. Mutant *sweet11;12* exhibited a reduction in primary root length to 1.70 cm when comparing root growth on 4% sucrose containing medium to medium without sucrose. The decrease in root length of the wild type Col-0 was 2.75 cm when comparing the two media (Figure 4A). The root phenotypes of Col-0 and line *sweet11;12* are shown in Figure 4C grown on vertical plates containing different sucrose concentrations (0, 2% or 4% respectively). Sugar levels were tested in roots of wild type Col-0 and *sweet11;12*. The sucrose concentration in *sweet11;12* roots was 36.43 mg/g, 87.45 mg/g and 109.81 mg/g respectively growing on media containing 0, 2% and 4% sucrose, and differed significantly from the values found in wild type Col-0 roots, being 41.67 mg/g, 69.25 mg/g, and 96.72 mg/g respectively (Figure 4B). Sucrose content in roots growing in sucrose containing medium showed significantly higher concentrations in *sweet11;12* roots than in Col-0 roots implying that the sucrose export and sucrose upward translocation were affected by SWEET11&12.

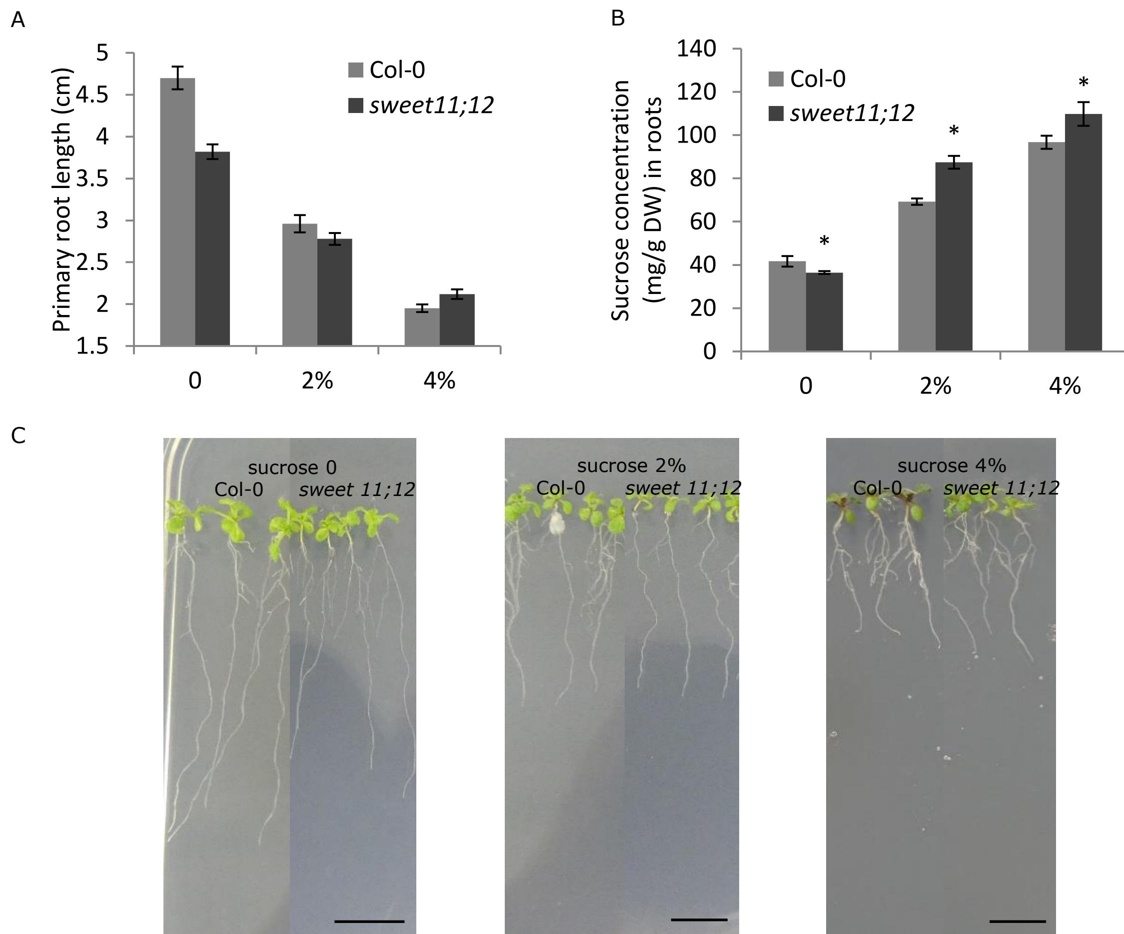


Figure 4. Primary root length and root carbohydrate concentrations of Arabidopsis *sweet11;12* line and wild type Col-0. A, main root length of Col-0 and *sweet11;12* grown on vertically oriented solidified medium plates containing sucrose at 0, 2%, and 4% respectively (mean ± SEM). B, the concentrations of sucrose in roots from Col-0 and line *sweet11;12* grown on 0, 2% and 4% sucrose medium. C, the root phenotype of Col-0 and line *sweet11;12* grown on vertical plates containing sucrose 0, 2% or 4% separately. The scale bar presents 1 cm. (mean ± SEM, n = 3-4 pools of mixed roots). Asterisks showed significant difference ($P<0.05$).

Expression of SWEET11 and SWEET12 in root tissues

To monitor the effects of sucrose on SWEET11 and SWEET12 expression in roots *in vitro,* a histochemical staining was performed on roots of transgenic Arabidopsis plants, equipped with either a SWEET11-GUS gene combination driven by its native promoter, or a SWEET12-GUS combination driven by its own native promoter, grown on media containing different levels of sucrose being 0, 2%, 4% and 6% respectively. The staining observed in the leaves of SWEET11-GUS line (Figure 5A) and SWEET12-GUS line (Figure 5B) proved the true transgenic nature of the lines, the expression of the fused protein products and the efficacy of the GUS staining protocol. Following up on this, both SWEET11 and SWEET12 were expressed in roots at sucrose 2% (Ⅱ), 4% (Ⅲ) and 6% (Ⅳ) while not or very slightly expressed in roots when sucrose was absent (Ⅰ). This indicated that the function of SWEET11 and SWEET12 in roots *in vitro* might depend on the presence of sucrose. It has been found earlier that expression of sugar transporters are modulated by sugar levels (Williams et al., 2000). The spatial expression patterns of both genes seemed to be restricted to the vascular strands in the roots (Figure 5C and 5D).

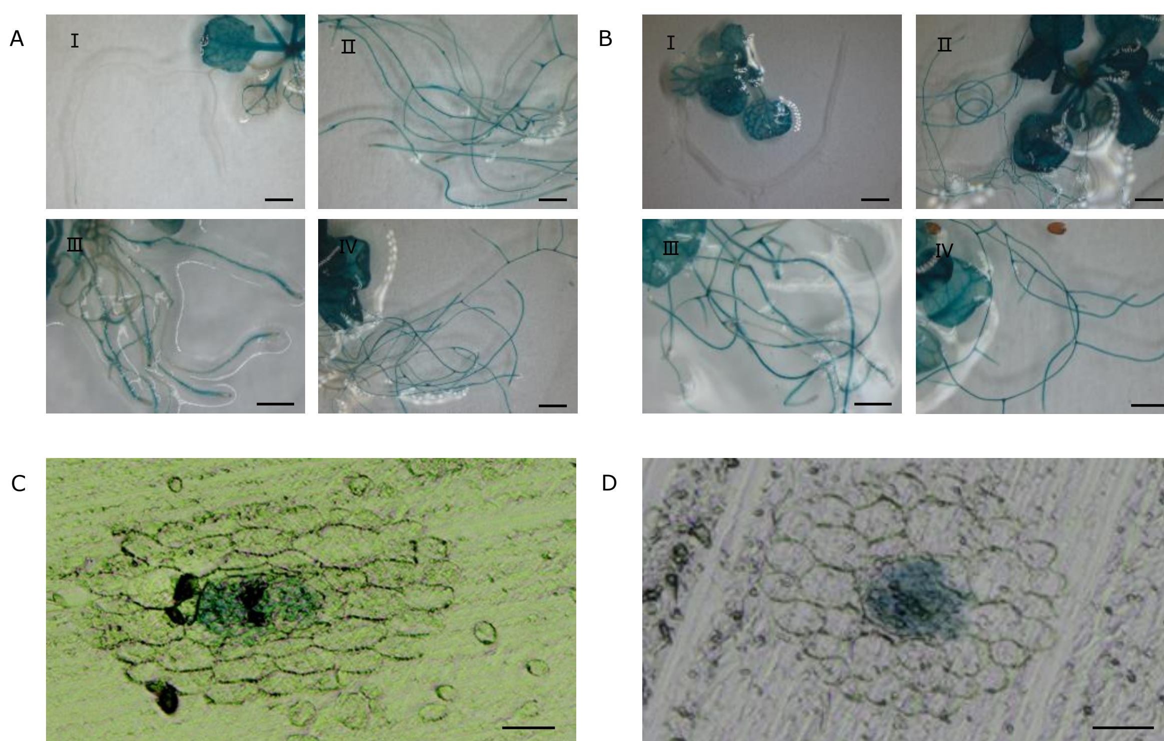


Figure 5. Histochemical analysis of GUS expression patterns of *p*AtSWEET11:AtSWEET11-GUS and *p*AtSWEET12:AtSWEET12-GUS in Arabidopsis lateral roots. The effects of sucrose concentrations 0, 2%, 4% and 6% on expressions of SWEET11-GUS (A) and SWEET12-GUS (B) fusion proteins are shown in Ⅰ, Ⅱ, Ⅲ, Ⅳ; for each Ⅰ = 0 sucrose; Ⅱ = 2% sucrose; Ⅲ = 4% sucrose; Ⅳ= 6% sucrose. Scale bars indicated 1 mm in Ⅰ, Ⅱ, Ⅲ and Ⅳ. Root cross sections of SWEET11-GUS and SWEET12-GUS grown in 2% sucrose containing medium are presented in C and D, respectively. Scale bar, 30 μm in C and D.

Growth characteristics of excised roots as affected by the *sweet11;12* double mutation

In the previous section it was found that higher amounts of sucrose accumulated in the roots of the double mutant *sweet11;12*. In general sucrose is synthesized in leaves and translocated in stems downwards to roots. To preclude the possibility that endogenous sucrose influences the activity of roots in exogenous sucrose uptake and root development, the growth of excised isolated Arabidopsis roots was studied. To compare root development of wild-type Col-0 with that of the *sweet11;12* mutant growth of these excised roots was monitored, as well as the medium pH value as indicator for active growth (Figure 6A). The medium pH showed a notable drop in Col-0 roots from 5.7 to 4.37 whereas in the *sweet11;12* root culture medium the pH drop was significantly smaller, from 5.7 to 4.53 (Figure 6B). This could indicate that possibly inferior nutrient assimilation was occurring in the roots of the double mutant, resulting in smaller pH drop and slower growth. The fresh weight was also compared after 2 weeks of culture: Col-0 had a biomass of 331.78 mg whereas *sweet11;12* had a weight of 167.16 mg (Figure 6C). Apparently, the isolated root growth of *sweet11;12* was impaired in liquid culture conditions, indicating that the SWEET11 and SWEET12 genes might be involved in exogenous sucrose assimilation in the wild type.

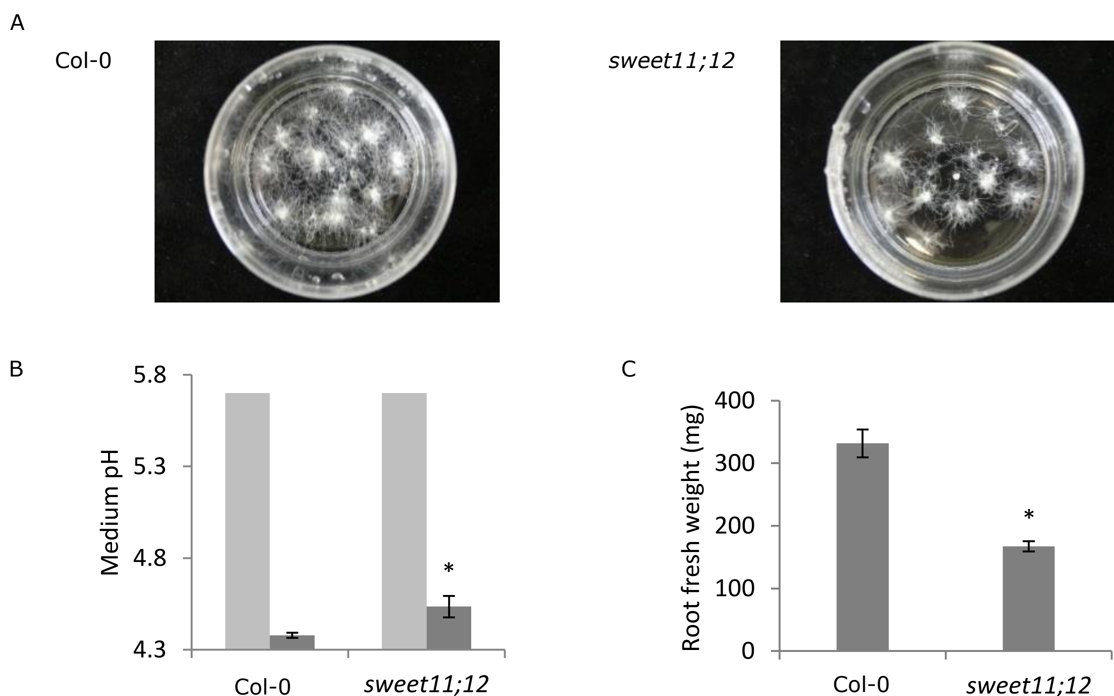


Figure 6. Growth characteristics of isolated roots excised from wild type Arabidopsis Col-0 and the double mutant line *sweet11;12* in root culture. A, the isolated root cultures of Col-0 and *sweet11;12* after 2 weeks. B, medium pH shift in Arabidopsis root cultures of Col-0 and *sweet11;12* (mean ± SEM, n = 6) in 2 weeks. Pale column, medium pH at the start pH 5.7; dark column, medium pH after 2 weeks. C, fresh weights of isolated roots of Col-0 and *sweet11;12* after 2 weeks of culture (mean ± SEM, n = 6). Asterisks shows differences significant at P<0.05.

Uptake of the esculin by protoplasts

To investigate the possible involvement of the SWEET genes in sucrose transmembrane translocation in more detail, the uptake of the fluorescent sucrose analogue, esculin, which is often used as phloem-mobile probe substituting sucrose, was monitored in both mesophyll protoplasts and in root protoplasts isolated from Arabidopsis *in vitro* grown seedlings. The esculin fluorescence intensity was assumed to be an indirect measure of the level of sucrose that could be assimilated in protoplasts. Protoplast fluorescence was monitored after half an hour incubation in 2 mM esculin W5 solution after which root protoplasts of *sweet11;12* mutant were compared to protoplasts of Col-0 serving as a control for uptake level (Figure 7A). This was to investigate a role of SWEET11 and SWEET12 in mediating sucrose (esculin) uptake in root protoplasts but no significant difference was observed. Root protoplasts of Col-0 displayed a fluorescence intensity at a similar level as *sweet11;12* (Figure 7B). Mesophyll protoplasts were also compared between the *sweet11;12* mutant and Col-0 and no significant difference could be found here, too (Figure 7C, 7D). In protoplasts, translocators SWEET11&12 which are supposed to be present in the membranes, did not seem to significantly affect esculin uptake.

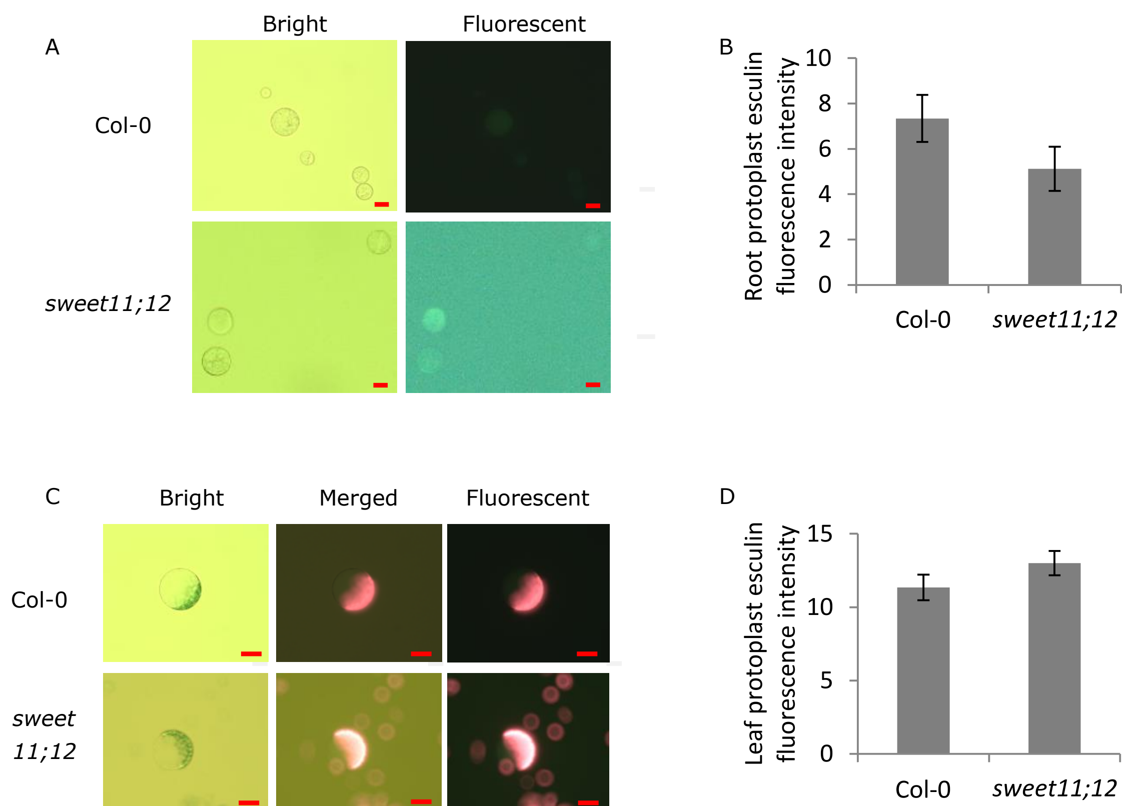


Figure 7. Esculin uptake in protoplasts of Arabidopsis root cells and mesophyll cells. Esculin fluorescence was seen in cyan with this filter combination whereas chlorophyll autofluorescence would show up as red. A, root protoplasts images of Col-0 and *sweet11;12* shown in bright field image and fluorescent field image. Scale bars present 20 μm. B, fluorescence intensity as determined in Col-0 and *sweet11;12* root protoplasts. C, mesophyll protoplasts images of Col-0 and *sweet11;12* shown from left to right in

bright field image, merged image and fluorescent field image. Scale bars present 20 μm. D, fluorescence intensity as determined in Col-0 and *sweet11;12* mesophyll cell protoplasts. Mean ± SEM, n > 20. No significant differences were found (P<0.05).

Discussion

Today, it is still not clear how exogenous sugar supplied through the medium is taken up, entering cells and subsequently being translocated upwards in plants in tissue culture. The applied sugar could be assimilated, migrated and utilized in roots or unorganized callus or wound tissues at stem bases *in vitro*. We tried to provide some preliminary answers to this translocation puzzle in the present study. We investigated the mechanism of sucrose translocation in *in vitro* grown Arabidopsis seedlings and selected the candidate transporter genes by comparing the expression of sucrose translocator genes in *ex vitro* grown plants to *in vitro* grown plants. As opposed to *ex vitro* plants where photosynthesis provides sucrose in leaves as the primary source tissue, the situation in *in vitro* plants is quite different because under such photomixotrophic or heterotrophic conditions, plantlets growth and multiplication mainly depend on the assimilation and utilization of exogenously supplied sucrose. Here, roots are supposed to play a role in sucrose uptake and translocation, acting as a source, which is the reverse of the "normal" *ex vitro* situation. The applied sucrose is assimilated by root border cells and afterwards likely imported into phloem cell tube for upwards translocation throughout the *in vitro* plant. Absorbed sucrose is possibly transferred in a radial pattern in the roots and then further distributed upwards through the phloem network for long distance translocation. Up till now, research on the related molecular mechanisms and physiological processes of the movement of exogenously supplied sucrose in roots *in vitro* has been scarce, in particular, on the role that sugar transporter genes play, and whether/how these genes could regulate sucrose uptake from the medium into epidermis/exodermis, and how sucrose is translocated upwards *in vitro*. However, the entire process is very complex and it will be very difficult to unravel all aspects and all details of the sucrose migration process from the site of uptake to the phloem, or how the cells and genes involved mediate cellular influx and efflux which are required for the apoplastic route. The results we obtained here attempted to offer some clues to the processes and mechanisms of exogenous sucrose migration in roots *in vitro* using the transgenic Arabidopsis *sweet11;12* double mutant line.

Expression pattern under *ex vitro* and *in vitro* conditions

It was found that sugar transporter genes show a tissue specific expression and thus we looked at the expression patterns of sucrose transporters SUCs and SWEETs in different tissues and under different growth conditions (Figure 1). Under *ex vitro* conditions, SWEET11 was more abundant in leaves as well as SUC2. Such an expression profile is consistent with the research reporting about sucrose influx and efflux in leaves where the translocation genes might be involved (Chen et al., 2012). We found SUC3 was abundant in leaves and roots but SUC4 was predominantly expressed in roots *ex vitro*. Our results here with respect to the low expression of SWEET16 and SWEET17, is contrary to earlier observations that both genes were expressed at intermediately high levels in roots of soil-grown plants (Guo et al., 2013). Under *in vitro* conditions, SWEET11 and SWEET12 were more expressed in leaves compared to other transporter genes. SWEET16, SWEET17, SWEET11, SWEET12, SUC3 and SUC4 showed higher expression levels in roots *in vitro*. Although studies are very limited on sugar transporter gene expression as

well as their putative function in tissue culture, we assume SWEET11, SWEET12, SUC3 and SUC4 might have important physiological functions in the process of sucrose uptake by the roots and upwards translocation under tissue culture conditions.

SWEET17 is thought to function as a major vacuolar fructose transporter regulating fructose content and mediating fructose transport across the tonoplast (Chardon et al., 2013; Guo et al., 2014). Similarly SWEET16 functions as vacuolar transporter in roots and mediates germination, growth and stress tolerance (Klemens et al., 2013; Guo et al., 2014). The sucrose uptake and efflux across cellular membranes are probably not related to SWEET16 and SWEET17. SUC3 and SUC4 are relatively highly expressed in roots at *ex vitro* and *in vitro* conditions. SUC3 and SUC4 are sucrose transporters in sieve elements and plant sink sites (Eckardt, 2003; Schulz et al., 2011; Duan et al., 2014) but are not our focus in this report. We assume that SWEET11 and SWEET12 are involved in the sucrose efflux process from source cells into the apoplast at *ex vitro* and *in vitro* conditions. In *ex vitro* the source cells are the mesophyll photosynthesizing cells in the leaves and the sucrose goes from the leaf parenchyma cells downwards through the phloem to the sink cells. In *in vitro* conditions, the source cells are the root cells and the sucrose has to be translocated upwards to the sink cells, presumably also through the phloem where the direction of the sap stream is reversed. The translocation via the apoplast route is driven by molecule diffusion and is associated with high concentrations of soluble sugars. SWEET11 and SWEET12 revealed higher expression levels in roots *in vitro*. Movement of sucrose in apoplast cell walls might be facilitated by transmembrane influx and efflux regulated by SWEET 11&12 in roots. If the transmembrane sugar flux via SWEET11 and SWEET12 proteins would be impaired, this could affect sucrose amounts in roots and subsequent growth *in vitro*. Our further studies provided indications for this using an Arabidopsis mutant in SWEET11 and SWEET12.

Growth profile of sweet mutant lines *ex vitro* and *in vitro*

We hypothesized that SWEET11 and SWEET12 exert an influence on plant growth *in vitro* and thus we were interested in the growth phenotype of *sweet11* and *sweet12* knock-out mutants *ex vitro* and *in vitro*. Arabidopsis knock-out mutant lines *sweet11* and *sweet12* grown *ex vitro* did not display morphological alterations (Figure 3) which was consistent with the outcome described by Chen et al. (2012). However, the double mutant line *sweet11;12* showed a clearly smaller size relative to Col-0 *ex vitro*. Such a growth phenotype was similar to the previous *ex vitro* study in which Chen et al. (2012) observed a 20% to 30% reduction of *sweet11;12* rosette diameter. The growth profiles of sweet mutants *in vitro* have not been investigated earlier. Here, the sizes after a period of growth of the single mutant lines *sweet11* and *sweet12* were similar to that of Col-0 *in vitro* whereas the double mutant *sweet11;12* lagged behind in growth (Figure 3). SWEET11 and SWEET12 might share the same role in translocation and compensate each other when one of them is non-functional; the identity score and similarity of the two is 83.8% and 90.0% respectively and this might support our supposition. It has been reported that SWEET genes function redundantly and several paralogs are closely related in translocation functionality (Eom et al., 2015). Arabidopsis SWEET genes show high similarities in amino acid sequence and have large similarities in transportation function (Chen et al., 2012; Chen et al., 2015a). It is still likely that SWEET11 and SWEET12 genes can be complemented by other related and quite similar SWEET genes but they do not seem to do so to the full extent as we observed a different growth profile in the double mutant.

Under *in vitro* conditions, most sucrose is thought to be taken up from the sucrose supplemented medium and transported upwards to the leaves as the sink site. We hypothesize that part of the sucrose translocation process is mediated by SWEET11&12. We investigated this further in *sweet11;12* roots of *in vitro* cultured plants in a biochemical way.

Primary root growth and sucrose contents of *sweet11;12* roots

To understand sucrose translocation in roots and the role played by SWEET11&12 in plant tissue culture, the *sweet11;12* root growth profile was analysed on media supplemented with varying sucrose concentrations (Figure 4). First, the primary root length between Col-0 and *sweet11;12* was compared. It was found Col-0 had a much greater difference of root length, comparing growth on medium lacking sucrose to medium with sucrose added to it. This difference was not so big in *sweet11;12*: here, the primary root length in the absence of sucrose was already smaller than the Col-0 control but the addition of sucrose did narrow the gap. At 4% the root length dropped further but both in Col-0 and in the double mutant. This observation for *sweet11;12* mutants at 1% and 2% was made earlier by Chen et al. (2012). Also *suc2* mutant seedlings had smaller roots than the wild-type in medium lacking sucrose (Gong et al., 2015). The primary root length being inhibited by sucrose addition might be due to the physical process of sugar hunting. In our *in vitro* experiments this means that the primary root length is an indication for the capability of "looking for sucrose". The length reduction seen when sucrose is supplemented in the medium then indicated a situation where the sucrose availability became adequate. Another explanation could be that the osmotic pressure caused by increasing sucrose in media inhibited root growth *in vitro*. The root biomass of *Hypericum perforatum* L. was reduced in higher sucrose concentrations, such as 5% or 7% compared to 3% sucrose (Cui et al., 2010). They argued that the observed reduced root biomass was caused by the higher osmotic potential of the media (Cui et al., 2010). Second, we looked at the levels of sucrose and found that sucrose accumulated more intensively in roots of the *sweet11;12* line grown on sucrose containing media relative to Col-0. The more intensively accumulated sucrose indicated that the sucrose translocation in a radial direction has been partly blocked in *sweet11;12*. These results indicate that sucrose translocation in radial direction is possibly mediated by SWEET11&12, particularly in the apoplast pathway joined with transmembrane flow. It is important to note that *sweet11;12*, which showed reduced sucrose transport, might also have a lowered sucrose influx through membranes *in vitro* inhibiting phloem uploading.

Localization of SWEET gene expression in *in vitro* roots

Plants growing in tissue culture are semi-autotrophic and therefore plants require exogenous sucrose supplementation and translocation to meet the demands for the buildup of carbon skeletons and for energy utilization (Hazarika, 2003). It is still not clear how the different transporter proteins mediate sucrose delivery *in vitro* whereas our analysis of tissue specific expression and subcellular localization clearly implicate a physiological role of sucrose transporters in tissue culture. In e*x vitro* conditions*,* SWEET proteins are localized in varying cellular sites, e.g. four clade III SWEET genes are found localized on the plasma membrane including SWEET11 and SWEET12 (Seo et al., 2011; Lin et al., 2014; Chen et al., 2015a). SWEET 1, 8, 9, 15 are localized in the plasma membrane (Seo et al., 2011; Kryvoruchko et al., 2016); SWEET16 and SWEET17 are confined to the vacuole (Chardon et al., 2013; Klemens et al., 2013; Guo et al., 2014);

SWEET9 and SWEET15 are at the Golgi cellular compartment (Lin et al., 2014; Chen et al., 2015b). With respect to the preferred sugar to be carried SWEET genes in clade I and II prefer to transport hexoses; clade III representatives (SWEET11, SWEET12) are efficient sucrose transporters; clade IV SWEET genes (SWEET16, SWEET17) transport fructose over the tonoplast membrane (Eom et al., 2015). The potential sucrose translocation might be elucidated by looking at where expression is localized. Analysis of GUS expression patterns in *in vitro* grown transgenic lines equipped with *p*AtSWEET11:AtSWEET11-GUS (or *p*AtSWEET12:AtSWEET12-GUS) fusion proteins revealed that SWEET11 (and SWEET12) were abundantly expressed in leaves on the one hand, and were also highly expressed in roots in the presence of sucrose on the other hand, especially in vascular tissues (Figure 5). This suggested that SWEET11 and SWEET12 might be induced by sucrose present in the medium, facilitate sucrose assimilation, and participate in phloem loading in the vascular system *in vitro*.

For heterotrophic roots the carbon source sucrose can diffuse through the apoplast pathway which consists of the inter-connecting cell walls and intercellular spaces. Apoplastic flow is driven by concentration gradients and thus logically inefficient and slow. Having passed through the cortex and endodermal cells, sucrose reaches the Casparian strip in root tissue. The Casparian strip is a barrier filled with lipophilic components and apoplastic flow is blocked there (Doblas et al., 2017). Sucrose molecules have to be taken up across membranes by sucrose transporters, particularly SWEET11 and SWEET12. Our proposed theory is that, also *in vitro* the apoplastic barrier, formed by Casparian strips and localized deposition of other aliphatic substances such as suberin and/or lignin, is present and affects radial translocation; as we suppose, sucrose translocation in this area is done by proteins on the cell membranes forming a sucrose channel regulating sucrose influx and efflux to and from a cell. However, to verify this hypothesis more research is still required to disclose the details of how SWEET11 and SWEET12 mediate sucrose loading in roots.

Excised isolated root growth in liquid medium

To exclude the influence of any photosynthesized sucrose and to understand the process of exogenous sucrose assimilation in the roots, a root culture was established for the *sweet11;12* mutant and the Col-0 control. Excised root sections which had lateral root meristems generated new adventitious roots in root culture; this growth of isolated roots depended exclusively on uptake and utilization of external sucrose. Roots from the double mutant *sweet11;12* in liquid medium showed a reduction in fresh biomass production as well as a minor change of pH compared to roots derived from Col-0 indicating an impairment in growth (Figure 6). Here, the pH shift was suggested to be a measure for growth in an isolated culture. The drop in medium pH is thought to be related to root growth, acidic metabolites release and element uptake (Butcher and Street, 1964; Flores et al., 1987; Felle, 1998; George et al., 2008a; George et al., 2008b). The release of protons in exchange for ammonium ions taken up results in medium being more acidic (George et al., 2008b). Mutant *sweet11;12* showed minor pH alteration and lower fresh weight probably because of inferior sucrose consumption. We suppose that SWEET11 and SWEET12 genes in the wild are involved in heterotrophic sucrose acquisition in an isolated root culture system.

Uptake of the sucrose analogue esculin in protoplasts

To understand whether SWEET genes regulate sucrose flux over membranes, isolated *sweet11;12* mutant protoplasts were incubated in liquid medium containing esculin, the fluorescent sucrose proxy coumarin β-glucoside, in order to show by measuring the intensity, the level of assimilated esculin. In our experiments, the *sweet11;12* mesophyll protoplasts displayed a similar fluorescence intensity compared to the Col-0 control protoplasts, indicating that esculin uptake was not reduced (Figure 7). Similar results were obtained with root protoplasts of *sweet11;12* relative to root protoplasts of Col-0 (Figure 7). However, we cannot be sure that the lack of any difference in esculin uptake by protoplasts is due to SWEET11&12 or other reasons. It is important to note that the protoplast esculin uptake approach is complex and sometimes ambiguous. First, various types of protoplast were extracted from different structures. These protoplasts, including vasculature protoplasts, stomata cell protoplasts, epidermis cell protoplasts and palisade/spongy mesophyll protoplasts, might have different capabilities for esculin uptake and might show esculin fluorescence levels in a wide range. Second, quite a few protoplasts easily burst in our procedures. The dead protoplasts might pose a bias on tested results. Third, the assay is not quantitative as the kinetic value and initial rates of uptake are not easy to determine (Gora et al., 2012). Still, some reports showed positive results using protoplasts esculin uptake. Rottmann et al. (2018) found that the esculin uptake in Arabidopsis protoplasts was mediated by SUC2. Tonoplast monosaccharide transporters TMT1 and TMT2 regulated esculin loading into the vacuole in Arabidopsis (Schulz et al., 2011). It has been found that all type I SUCs tested so far, type II SUCs of dicotyledons, and sucrose-specific SWEETs can take up esculin in plant species protoplasts (Gora et al., 2012; Nieberl et al., 2017; Rottmann et al., 2018). Protoplasts expressing SUC3 accumulated esculin in their cytoplasm whereas yeasts expressing SUC3 did not (Gora et al., 2012; Rottmann et al., 2018). The fast esculin approach has been used to confirm functionality of many different sucrose transporters (Rottmann and Stadler, 2019). However, we were unsuccessful in confirming in this way the capability of SWEET11 and SWEET12 genes in sucrose uptake over the membranes.

Conclusion

In plant tissue culture, the sucrose transporters SWEET11 and SWEET12 are found to play an important role in sucrose translocation from the medium being the source, through the roots to other parts of the plants being sinks. In particular, the most likely sucrose transport route *in vitro* might begin directly at the interface between medium and root, through the apoplast, and into endodermis and sieve element-companion cell complex. SWEET11 and SWEET12 possibly might be involved in sucrose export by cellular radial transport and the subsequent translocation into the vasculature. Boosting the efficiency of the sucrose mobilization from the medium into the plants by improving or expanding the expression or performance of sucrose translocator genes might contribute to improved growth of *in vitro* propagated plants or a decreased recalcitrance of specific genotypes or species.

Supplemental table list of primers used in the PCR analysis

Gene target	Primer sequence (in 5'-- 3' order)	Accession no.
SWT1-F	CTTCTCCACTCTCCATCATGAGATT	AT1G21460
SWT1-R	CATCTGCAGATTTCTCTCCTTTGT	
SWT2-F	AACAGAGAGTTTAAGACAGAGAGAAG	AT3G14770
SWT2-R	ATCCTCCTAAACGTTGGCATTGGT	
SWT3-F	CCAACTTTTCCCTAATCTTTGTTCTTC	AT5G53190
SWT3-R	AACACCCTTGAAAATGTTACTATTGGA	
SWT4-F	CCATCATGAGTAAGGTGATCAAGA	AT3G28007
SWT4-R	CAAAATGAAAAGGTCGAACTTAATAAGTG	
SWT5-F	TGACCCTTATATTTTGATTCCAAATGGT	AT5G62850
SWT5-R	GCCAAGTTCGATTCCAGCATTC	
SWT6-F	GACTCGGTTACGTTGGTGAAGT	AT1G66770
SWT6-R	CAAACGCCGCTAACTCTTTTGTTTAA	
SWT7-F	GACCCATTCATGGCTATACCAAAT	AT4G10850
SWT7-R	ATCCCATAATCCGAAGTTTAATAACACT	
SWT8-F	TTGCTCTCTTCTTCATCAATCTCTCT	AT5G40260
SWT8-R	AGATCCTCCAGAAAGTCTTCGCT	
SWT9-F	GCAAGAGAAAGAGAGAAAAGTGAAGA	AT2G39060
SWT9-R	CCCATAAAACGTTGGCACTGGT	
SWT10-F	TAGAGGAAGAGAGAGGGAGAGAGT	AT5G50790
SWT10-R	ATATACGAACGAACGTCGGTATTG	
SWT11-F	TCCTTCTCCTAACAACTTATATACCATG	AT3G48740
SWT11-R	TCCTATAGAACGTTGGCACAGGA	
SWT12-F	AAAGCTGATATCTTTCTTACTACTTCGAA	AT5G23660
SWT12-R	CTTACAAATCCTATAGAACGTTGGCAC	
SWT13-F	CTTCTACGTTGCCCTTCCAAATG	AT5G50800
SWT13-R	CTTTGTTTCTGGACATCCTTGTTGA	
SWT14-F	ACTTCTACGTTGCGCTTCCAAATA	AT4G25010
SWT14-R	CAGTTCAACATTAAAGTCAATCACTAATTC	
SWT15-F	CAATGACATATGCATAGCGATTCCAA	AT5G13170
SWT15-R	GGACTCATCACGACAATACTCTTAAG	
SWT16-F	GAGATGCAAACTCGCGTTCTAGT	AT3G16690
SWT16-R	GCACACTTCTCGTCGTCACA	
SWT17-F	AGTGACAACAAAGAGCGTGAAATAC	AT4G15920
SWT17-R	ACTTAAACCGTTGCTTAAACCAACC	
SUC1-F	AGAGACACAGTCGCCGGA	AT1G71880
SUC1-R	AAAGGAGTACTGAAAGTAATAGCTAATGGG	
SUC2-F	CCGGAACGGCTTCGTAAGA	AT1G22710
SUC2-R	GATTCCGAGTAGCTGCACGTAAG	
SUC3-F	CAAGAACCGCAGCCGTAATC	AT2G02860
SUC3-R	CTTGACCGCCACCGGAAT	
SUC4-F	AGTGTCAAGCGAGGAACGCATA	AT1G09960
SUC4-R	AGTCACACGAGAAGCCATTGC	
SUC5-F	GGGCTATGGGATTCCATTAG	AT1G71890
SUC5-R	TAAAAGACAGACGACCAAGG	
SUC6-F	TCCTGTCTCCGGCCTGCTT	AT5G43610
SUC6-R	AGGCGCCCATAGCGATGA	

SUC7-F	GTCTTTAAGAGACAAGCCCAC	AT1G66570
SUC7-R	AGACTGTCTATCCACAGTCGT	
SUC8-F	CTAGCTTCCATAATCTCAAGT	AT2G14670
SUC8-R	TTGGTAAGTTTCCACCTCCAAAA	
SUC9-F	GTGGTTCCTGATGAGCCG	AT5G06170
SUC9-R	GAGAAGCTGAACGTATGGG	
UBQ10-F	CACACTCCACTTGGTCTTGCGT	AT4G05320
UBQ10-R	TGGTCTTTCCGGTGAGAGTCTTCA	
Actin2-F	TCTTAACCCAAAGGCCAACA	At3G18780
Actin2-R	CAGAATCCAGCACAATACCG	

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CHAPTER 4

The AtPIP1 aquaporins regulate root activity and plant growth in tissue culture

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Abstract

Plant growth *in vitro* as well as its multiplication is greatly determined by medium nutrient uptake and water hydraulic conductance. Water molecules might migrate into plant tissues by diffusing via porous cell walls, the apoplast, or by moving from cell-to-cell where the transcellular water flow is modulated by water channels, i.e. the aquaporins. However, the significance of cellular water flow remains ambiguous in plant growth *in vitro* and as a consequence also the function of aquaporins. Our study identified the contribution of plasma membrane intrinsic proteins, PIPs subfamily PIP1, to plant growth through investigating physiological characteristics of miRNA-induced-PIP1s-silenced plants *in vitro*. We found that PIP genes are indeed expressed in *in vitro* roots but that expression of aquaporin PIP1s is lower after increasing the level of sucrose in media indicating a negative effect of exogenous sucrose on transmembrane water regulation *in vitro*. To further study the correlation between water translocation and exogenous sucrose translocation we looked at plants impaired in PIP1s expression, so in cellular water translocation. The PIP down-regulated mutants used showed a reduction in size and a reduction in biomass accumulation but an increase in the sucrose content maintained in *in vitro* roots compared to wild type Col-0. The roots of miRNApip1 plants, excised and cultured separately, displayed reduced growth *in vitro* suggesting that the PIP1 gene subfamily in the wild type might be vital for nutrient assimilation in roots probably through mediating membrane water flow, but not for the earlier observed sucrose accumulation in roots. The elucidation of the precise mechanism and relevance of these findings requires further studies. After establishing the role of PIP1 in *in vitro* roots, we investigated its possible function in higher plant parts, such as the *in vitro* leaves. Transpiration was monitored by using a leaf water loss assay but no difference in transpiration was observed between wild type and silenced plants *in vitro*. The role of the PIP1 gene family, engaged in cellular water translocation, in transpiration might be negligible *in vitro*. In conclusion, our experimental results confirmed the importance of PIP1s by revealing that PIP1 genes are expressed and indirectly mediate sucrose levels in roots and that PIP1s regulate plant growth *in vitro*. This characterization of PIP1s in plant tissue culture provides a first glimpse on growth mechanisms and related physiological processes that could be exploited in optimizing plant growth. Our research will contribute to plantlet regeneration and multiplication by stimulating more efficient water transportation and enhanced sucrose translocation in *in vitro* plants.

Keywords: *Arabidopsis thaliana* growth, aquaporin PIP1 genes, root water transport, tissue cultured plants

Introduction

Plants require various indispensable nutrients to grow, develop and reproduce, such as a carbon source, water and minerals. Carbon originates from the CO_2 in the air which is used in specialized plant organs, i.e. the leaves, where light energy is converted into chemical energy stored in carbohydrates by a photosynthetic reaction (Barber, 2002; Mazor et al., 2012). Water and ionic nutrients are assimilated in roots in concentric rings of cell layers (Steudle and Peterson, 1998). Mineral assimilation is characterized by selectivity of transport and accumulation in roots (Marschner, 2011). Water is not taken up actively, but moves into roots driven by a water potential gradient caused by transpiration pull. Water travels upwards to the shoots, finally arriving at the apex through the xylem which consists of dead empty vessels (Frensch and Steudle, 1989). This axial water translocation knows hardly any barriers to water movement whereas radial water transport over relatively short distances is complex and more difficult across concentric cell layers (Doussan and Vercambre, 1998; Knipfer and Fricke, 2010a). In the radial pathway water has to move across the epidermis, the cortex including exodermis and endodermis, and finally arriving at the central stele.

Radial water flow to the root xylem vessels involves three parallel paths: symplastic, coupled transcellular and apoplastic respectively (Steudle and Peterson, 1998). The symplastic pathway involves water moving through plasmodesmata which provides cytoplasmic continuity between neighbour cells (Burch-Smith and Zambryski, 2012). The efficiency of symplastic transport is associated with the aperture size and exclusion limit of the plasmodesmata and the number of plasmodesmata (Roberts and Oparka, 2003; Sager and Lee, 2014). The transcellular or transmembrane pathway represents water moving through plasma membrane and cytoplasm. Influx water transporters and efflux water transporters carry water from one cell to the other. The symplastic pathway is difficult to distinguish in experiments from the transcellular pathway mediated by aquaporins (Li et al., 2014). The sum of symplastic and transcellular pathways is named as cell-to-cell pathway (Steudle and Peterson, 1998). The apoplastic pathway consists of water movement through cell wall pores and free intercellular spaces. However, the passive flow of water in the apoplast can be interrupted by "cell wall discontinuity" (De Rufz de Lavison, 1910) in the endodermis. This is because of the presence of the Casparian strip, which blocks free passage of water and minerals, and other water blocking structures in the apoplastic space, such as lipophilic suberin deposition (Barberon, 2017). In some plant species, microscopic analysis has shown that barriers to radial water movement are formed in the exodermis of roots (Shiono et al., 2014) where suberin and/or lignin are deposited. The influx and efflux transmembrane water transport are necessary to facilitate water movement across the endodermis, replacing the blocked apoplastic pathway. Therefore, water transporters (aquaporins) are required in roots in particular at the exodermis and endodermis where apoplastic water transport has been interrupted.

Water channel proteins, called aquaporins, are small proteins comprising six membrane spanning α-helices connected by five loops (Murata et al., 2000) and they usually assemble as tetramers on membranes with each monomer functioning as independent water channel (Murata et al., 2000; Fetter et al., 2004; Bienert et al., 2012). Aquaporins facilitate or regulate the movement of water molecules, the flux into or out of a cell, along the water potential gradient. They facilitate not only the passage of water but also some small neutral molecules across membranes (Chaumont et al., 2005), such as gases

(CO_2, ammonia) (Kaldenhoff, 2012), reactive oxygen species (hydrogen peroxide: H_2O_2) (Bienert and Chaumont, 2014) and metalloids (boric acid, silicic acid, antimonite and arsenite) (Maurel et al., 2015). There are different water channel aquaporin isoforms, which have been categorized into plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), NOD26-like intrinsic protein (NIP), small basic intrinsic protein (SIP), unknown intrinsic protein (XIP) and hybrid intrinsic protein (HIP) (Johanson et al., 2001; Danielson and Johanson, 2008). NIP is proposed to control solute transport while PIP is described to be the main gateway mediating water permeability (Ma et al., 2006; Mitani-Ueno et al., 2011). The PIP family is divided into the subgroups PIP1 and PIP2. In fact Arabidopsis has 35 aquaporins, of which AtPIP1 group has 5 genes: PIP1;1 to PIP1;5, and AtPIP2 group includes 8 genes: PIP2;1 to PIP2;8 (Johanson et al., 2001). The two subfamilies PIP1s and PIP2s have a highly conserved amino acid sequence while functions might differ drastically (Chaumont et al., 2000). PIP2 proteins have been shown to facilitate primarily water translocation (Chrispeels et al., 2001) while PIP1 proteins regulate both CO_2 diffusion (Uehlein et al., 2003) and water permeability likely via conjunction with PIP2 proteins (Fetter et al., 2004). Cooperative expression of PIP1 and PIP2 isoforms leads to heterotetramer formation enabling water permeation in *Xenopus* oocytes (Li et al., 2016; Yaneff et al., 2016; Byrt et al., 2017). The water transport efficiency in leaves, named as hydraulic conductivity, is proposed to be regulated by aquaporins (Sack and Holbrook, 2006) and a correlation between PIP gene expression and dynamic leaf hydraulics have been reported (Cochard et al., 2007). PIP1;2 antisense expression accounted for reduction of membrane hydraulic conductivity of isolated protoplasts and decreased root hydraulic conductivity (Kaldenhoff et al., 1998; Martre et al., 2002). The suppression of PIP1 homologues resulted in reduction in water permeability of protoplast membranes, in a decrease in root hydraulic conductivity and a lower level of transpiration in tobacco (*Nicotiana tabacum*) (Siefritz et al., 2002; Siefritz et al., 2004). These findings indicated that PIP1 genes are associated with cellular water transport in *in vivo*. However it is unknown whether aquaporin PIP1 genes are involved in transcellular water flux, plant growth and root development in plant tissue culture. It is important to note that tissue culture conditions differ a lot from plant growth conditions in a field or greenhouse environment.

Plants growing *ex vitro* acquire energy and their carbon source from the aerial environment, and obtain water and dissolved inorganics from the soil by roots to sustain their growth and propagation. Sucrose synthesized in autotrophic plant leaves is actively loaded into the SE-CCC (sieve element/companion cell complex), leading to high sucrose content of the cell sap resulting in a high osmotic pressure in the sieve tube network compared to the surrounding cells (Geiger et al., 1973; Lemoine, 2000). For translocation sucrose travels through the phloem towards sink tissues such as shoot apices and roots (Aoki et al., 2012) along an osmotic turgor pressure gradient (Minchin and Lacointe, 2005; Thorpe and Minchin, 2017). However mixotrophic or heterotrophic plants growing *in vitro* take up carbohydrates from the gellified medium where exogenous sugar and other nutrients are abundant. The absorption and mobility of exogenous sucrose in roots can be influenced by, linked to or even dependent on the occurrence of water translocation. Apoplastic nutrient molecule movement is influenced by bulk water flow (Enstone et al., 2002; Barberon and Geldner, 2014). It could be that the uptake of solubilized sucrose moving passively or actively at the cellular level, e.g. sucrose translocator proteins, is linked to the water flow in roots *in vitro*. Aquaporins might regulate directly or indirectly apoplastic sucrose movement, particularly in case of apoplastic barriers. Studies on the potential action of water channels aquaporins in plant

tissue culture, with respect to aspects of exogenous sucrose uptake and root development are scarce. The success of *in vitro* plant tissue propagation, which is used in multiple crops on a commercial scale, but still faces recalcitrance and low quality in many others, relies on efficient transport of water and the assimilation of sucrose and other nutrients (Hazarika, 2006).

In this study we looked at the contribution of water movement along the radial pathway, as facilitated by PIP1s, to plant growth *in vitro*. The expression of both PIP1 and PIP2 subfamily genes was examined in roots at different concentrations of sucrose to investigate the correlation between water translocation and sucrose assimilation. To study the link between water translocation and plant growth we investigated whether silencing multiple PIP1 genes would lead to decreased plant growth in plant tissue culture and in isolated root cultures, through observing fresh weight and dry weight *in vitro*. The sucrose concentrations in roots were also determined in comparison to the wild type. We also started experiments to understand the influence of PIP1 on transpiration. Our results showed that PIP1 genes expression might not be directly linked to transpiration *in vitro* but PIP1 genes still are functionally correlated with exogenous sucrose uptake and plant growth in tissue culture conditions.

Materials and methods

Plant materials

The seeds of *Arabidopsis thaliana* lines miRNApip1a (also named as 35S:mir1-3) and miRNApip1b (also named as 35S:mir1-8) were obtained from Prof. M.Moshelion moshelio@agri.huji.ac.il of the Institute of Plant Sciences and Genetics in Agriculture, in the Hebrew University of Jerusalem (Sade et al., 2014). The entire PIP1 gene family, PIP1;1, PIP1;2, PIP1;3, PIP1;4, and PIP1;5, were targeted to be down regulated in miRNApip1a and miRNApip1b. One sequence of 21 nucleotides (nt), AGGTTCCGTAGCGAACCCGAT, introduced in endogenous miRNA164b backbone (Alvarez et al., 2006), was designed to silence the entire PIP1 gene subfamily in *Arabidopsis thaliana*. The vector pMLBART (Eshed et al., 2001) equipped with the modified miRNA164b containing the 21 nt consensus sequence under control of the 35S constitutive promoter was transformed to Arabidopsis ecotype Columbia (Col-0) plants (Sade et al., 2014).

Growth conditions

All seeds of Arabidopsis thaliana Col-0 and two lines miRNApip1a and miRNApip1b were sterilized by submersion in 70% (v/v) ethanol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 1 min followed by 2% (w/v) sodium hypochlorite solution (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 15 minutes, and rinsed in sterile deionized water three times, and finally placed on dishes with full strength Murashige and Skoog medium (MS; (Duchefa, Haarlem, The Netherlands) (Murashige and Skoog, 1962; Gamborg et al., 1976)) (pH 5.7-5.8) containing 1% (w/v) Daishin agar (Duchefa, Haarlem, The Netherlands), under darkness at 4 °C for 72 hours to synchronize germination. The germinated seeds were transferred to Petri dishes (10×10 cm) (Duchefa, Haarlem, The Netherlands) containing full strength autoclaved (121°C for 20 minutes) MS medium (Duchefa, Haarlem, The Netherlands), 0, 2% or 6% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 1% (w/v) Daishin agar (Duchefa, Haarlem, The Netherlands), pH 5.7-5.8 and grown in a climate chamber (Conviron BDR16)(Conviron Germany GmbH, Berlin, Germany) with parameter light intensity 50 μmol $m^{-2}s^{-1}$ (Philips Master TL-D 36)(Philips, Poland), 16 h light and 8 h dark photoperiod, and 22/18 °C light/dark temperature regime.

Expression analysis by quantitative PCR

Total RNA was isolated from Col-0, miRNApip1a and miRNApip1b seedlings using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was subjected to genomic DNA killing using RNase-free DNase I (Qiagen, Hilden, Germany). The reaction containing 1 μg RNA template, 1 μl 10X DNaseI reaction buffer, 1 μl DNaseI and sterile deionized water, was incubated at room temperature for 15 minutes. Later 1 μl 25 mM EDTA reaction mixture (Qiagen, Hilden, Germany) was added and incubated at 65 °C for 10 minutes to stop the reaction. cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The reaction mixture containing 4 μl 5X reaction mix, 1 μl reverse transcriptase, 1 μg RNA template, and milliQ water, was incubated in a PCR machine at 25 °C for 5 minutes, 46 °C for 20 minutes, 95 °C for 1 minute and held at 10 °C. The concentration of cDNA synthesized was diluted one-tenth for further utilization.

To investigate the transcriptional levels in miRNApip1a and miRNApip1b of all PIP1 genes quantitative real-time PCR (qPCR) was performed using CFX96TM real-time PCR system (Bio-Rad Laboratories, Hercules, USA). Three biological replicates were performed using 20 μl reaction volume which contained 2 μl cDNA, 10 μl 2X SYBR GREEN super mix (Bio-Rad Laboratories, Hercules, USA), 2 μl forward/reverse primer pairs of which sequence was provided by Sade et al. (Sade et al., 2014), and milliQ water. The qPCR program was 95 °C preheating for 3 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute, 95 °C for 10 minutes and temperature increment for 5 seconds. The relative gene expression level was calculated using method the $2^{-\Delta\Delta Ct}$ with UBQ-10 served as reference gene where C_t represents the threshold cycle (Livak and Schmittgen, 2001).

Growth measurements

Arabidopsis plants, wild type Col-0, miRNApip1a and miRNApip1b, were collected for weight analysis after 4 weeks of growth *in vitro*. Plants were carefully removed from the media, soaked in water to dispose of media adhering, and rubbed in tissue paper. These plants were subsequently placed in empty tubes of which the weight was already known and were weighed by 0.01 mg resolution with an analytical balance, XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands) to get the fresh weight. Subsequently, the plants were dried in an oven (Omnilabo International B.V., Breda, The Netherlands) at 70 °C overnight and were then weighed using a 0.01 mg resolution analytical balance again to establish dry weight.

Sugar extraction and determination

For 4-weeks-old plants of the wild type, miRNApip1a and miRNApip1b, leaves and roots were harvested and collected from at least 30 different plants per line at the end of a light cycle. The tissues were freeze dried overnight using (Freeze Dryer)(ilShin Biobase Europe B.V., Ede, The Netherlands) and weighed for later extraction. Soluble sugars were extracted using dried tissues and tested in High Performance Liquid Chromatography (HPLC) as described (Schneider et al., 2008). Each sample of 20 mg dry tissue was incubated at 80 °C in 2 ml 80% (v/v) ethanol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in an HLC thermoshaker (salmenkipp, Breukelen, The Netherlands) at a speed of 500 rpm for 40 minutes. After that the samples were centrifuged at 4000 rpm for 10 minutes in a Heraeus Multifuge 3L-R (Kenodo Laboratory Products, Asheville, USA); and then the supernatant was directly transferred to a new tube by a glass Pasteur pipette. The materials were extracted with 80% ethanol 3 times per sample. The supernatant was subsequently dried by vacuum using the RapidVap (Beun De Ronde, Abcoude, The Netherlands) and then the extracted residues were dissolved in MQ water. The soluble sugars in solution were separated by High Performance Liquid Chromatography with a Dionex column (Dionex ICS 5000)(Thermo Fisher Scientific B.V., Waltham, MA USA). The eluent was 500 mM NaOH and the process took 80 minutes. The chromatograms were analysed using the Chromeleon program (Dionex ICS 5000)(Thermo Fisher Scientific B.V., Waltham, MA USA), and the known oligosaccharide standard samples were compared to identify peaks. Using peak surfaces of standard curves sample sugar concentrations were calculated and shown as mg per dry sample (g).

Water loss assay

Rosette leaves, collected from *in vitro* grown Arabidopsis lines at the end of the fourth week, were kept at room temperature with the adaxial side down in open Petri dishes on

the bench. Leaves from five plants per line were excised, collected and subsequently weighed in petri dishes at time=0. Subsequently, the dishes were weighed using the XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands) at several time points after excision. The weight loss represented the water lost through leaf transpiration.

Isolated root growth

The primary roots were excised into 1-cm-fragments originating from 2-weeks-old cultured Arabidopsis seedlings. An identical number of root fragments taken from 10 plants per line were inoculated aseptically in one 125 ml Erlenmeyer flask (Duchefa, Haarlem, The Netherlands) containing 30 ml root culture medium (ARC) aliquots comprised of full strength MS including vitamins (Murashige and Skoog, 1962)(Duchefa, Haarlem, The Netherlands), 1% (w/v) sucrose (Duchefa, Haarlem, The Netherlands) and 0.29 μM indole-3-acetic acid (IAA)(Duchefa, Haarlem, The Netherlands), pH 5.75 (Czakó et al., 1993). All the flasks, 6 flasks per line, were covered by aluminium foil to ensure dark conditions and were incubated in a shaker at approximately 50 rpm in a climate chamber with the temperature set at 22 °C. Extensive adventitious roots were produced in 2 weeks and were subsequently collected for weight determination. The fresh weight of these adventitious roots was determined using the XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands). The root phenotype was photographed under light microscopy using an Axiophot light microscope (Carl Zeiss Microscopy, White Plains, United States).

Statistical analysis

All data were subjected to a one-way ANOVA together with the Bonferroni-Holm adjustment test or t test using the statistical package GraphPad Prism (GraphPad Software, San Diego, USA) or excel. Values are displayed as means ± SEM. All data were obtained from at least three independent replications. Asterisks above each bar represent statistically significant differences (*, $P<0.05$ and **, $P<0.01$).

Results

Aquaporin gene expression *in vitro*; effect of exogenous sucrose

A class of aquaporins, i.e. the plasma membrane intrinsic proteins (PIPs) is supposed to be involved in transmembrane water translocation *in vivo*. As part of our study on nutrient and water flow *in vitro* we investigated the expression levels in *in vitro* grown plantlets of the gene family members coding for two PIP proteins, i.e. PIP1 and PIP2. Concomitantly, we investigated the influence of sucrose on PIP gene expression using different concentrations. Measurable expression was detected for all PIP genes, except for PIP2;1, PIP2;4 and PIP2;5. Increasing the sucrose concentration in the medium lowered expression levels for most PIP genes, except for PIP2;6 and PIP2;8. The pattern of PIP2;2 was more complex, but at the highest sucrose concentration, expression was not increased. Indeed, all PIP1 subgroup genes were down regulated while PIP2 subgroup genes showed a more differentiated pattern. This indicated that sucrose might have a negative effect on cellular, transmembrane water translocation mediated by PIP1. We will focus further on the PIP1 function by investigating growth and the link between water translocation and sucrose translocation in mutants down regulated in PIP1 gene expression.

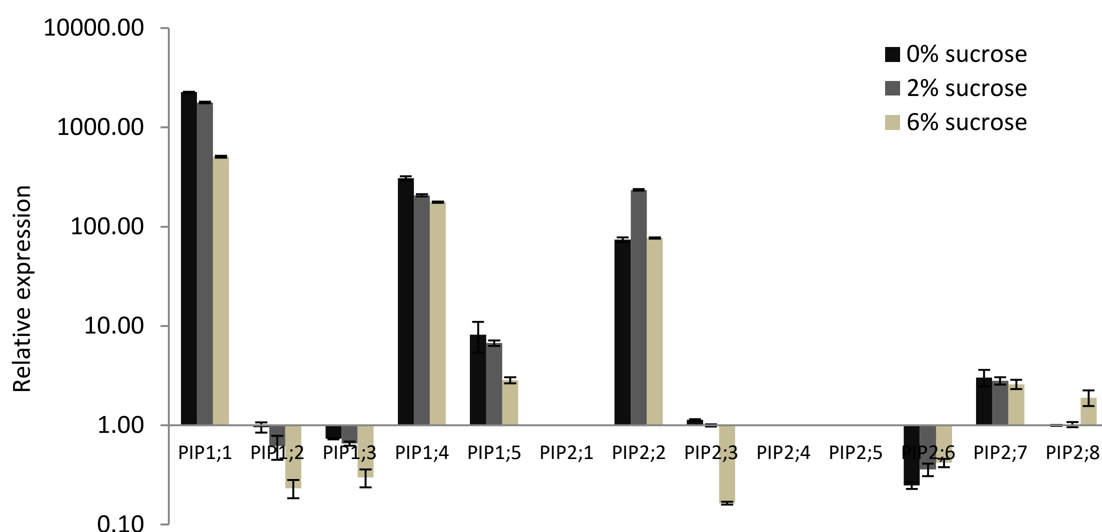

Figure 1. Relative expression of all PIP genes in roots of Arabidopsis Col-0 grown *in vitro* in medium containing sucrose at 0, 2% and 6% respectively (mean ± SEM). n = 3 pools of plants. The expression of the reference gene, Ubi-10, is set at 1.

PIP1 transcript levels in miRNApip1a and miRNApip1b plants

The PIP1 subfamily is reported involved in transport of water across plant cell membranes and root hydrostatic hydraulic conductivity in Arabidopsis *in vivo* (Siefritz et al., 2002; Postaire et al., 2010; Vajpai et al., 2018). To examine PIP1 gene functionality a modified miRNA164b (Alvarez et al., 2006) was made containing a 21-nucleotides insert, AGGTTCCGTAGCGAACCCGAT, under the control of the 35S constitutive promoter targeted to silence the entire PIP1 subfamily: PIP1;1, PIP1;2, PIP1;3, PIP1;4, and PIP1;5; thus, transformed and silenced Arabidopsis lines miRNApip1a and miRNApip1b were established (Sade et al., 2014). Our quantitative RT-PCR analysis of *in vitro* grown plants of both silenced lines showed a high expression of the synthetic miRNA164b-PIP1 (Figure

2A). We further examined the down-regulation of PIP1 and PIP2 genes in transgenic lines miRNApip1a and miRNApip1b compared to the wild-type Col-0 as control (Figure 2B). All PIP1 genes were expressed at significantly lower levels in miRNApip1a and miRNApip1b lines relative to wild type Col-0. However PIP1;1 and PIP1;4 were at lower expression levels in miRNApip1a (16% and 13%) than in miRNApip1b (30% and 31%). The miRNApip1b showed lower levels of PIP1;2 expression; and approximately the same level of PIP1;3 and PIP1;5 expression compared with miRNApip1a. On top of the reduction in expression of the PIP1 genes, both lines revealed significant reductions in expression of PIP2 genes, although not to the same extent as for PIP1. The PIP2 expression reduction varied from a slight reduction of 14% in PIP2;6 to a 50% reduction in PIP2;2 expression. This indicated that PIP2 genes might also serve as silencing targets for the modified miRNA164b molecule in these PIP1-down-regulated miRNApip1 lines.

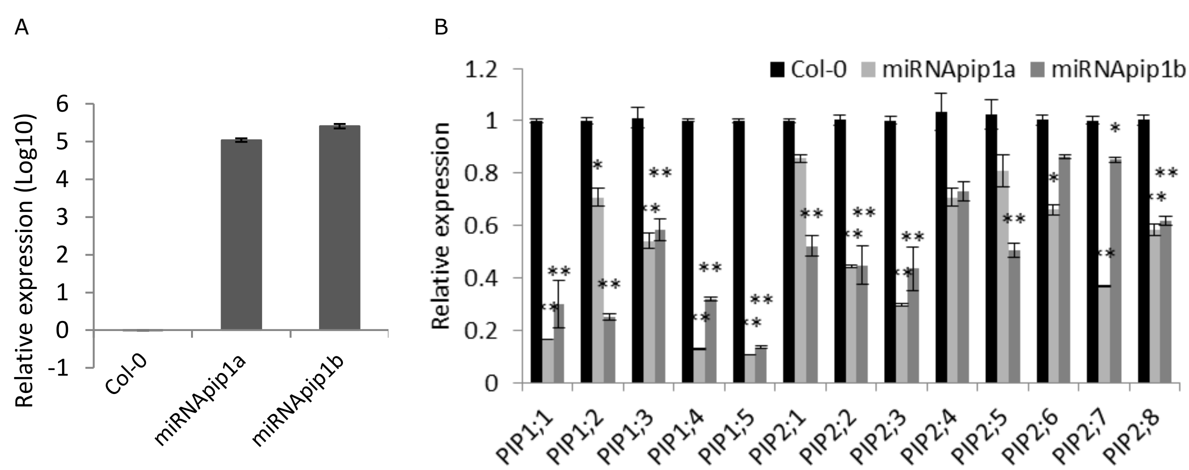

Figure 2. Molecular characteristics of the miRNApip1a and miRNApip1b transgenic plants. A, the expression (shown as log10 of original expression level) of miRNA164 in the transgenic lines and Col-0. B, PIP genes relative expression in Col-0, miRNApip1a and miRNApip1b lines. All PIP transcripts in the miRNApip1a and miRNApip1b plants were compared to wild type Col-0, set at 1 (mean ± SEM, n = 5-10 plants). Asterisks show significant differences: $P < 0.05$ (*) and $P < 0.01$ (**).

The effect of reduced PIP1 expression on growth *in vitro*

To study the effect of a reduction in PIP1 gene expression and consequently in water translocation on *in vitro* plant growth, the size and biomass accumulation, as fresh and dry weight, were measured in miRNApip1a and miRNApip1b plants. Visual observations showed a somewhat smaller plant size for the miRNApip1a and miRNApip1b plants when compared with wild type Col-0 (Figure 3A). MiRNApip1a plants had an average fresh weight of 60.14 mg, whereas miRNApip1b plants had an average fresh weight of 93.30 mg; both significantly lower compared to the average fresh weight of wild type Col-0, 275.24 mg (Figure 3B). The dry weights in miRNApip1a and miRNApip1b were also significantly reduced, 3.30 mg and 4.47 mg respectively relative to Col-0, 13.05 mg (Figure 3C). Apparently growth, illustrated by the fresh biomass and dry biomass, was impaired in miRNApip1a and miRNApip1b plants. When growth is considered to be dependent on nutrient availability, our results indicated that PIP1 genes possibly might have an indirect influence on sucrose accumulation or translocation *in vitro*. It has previously been reported that aquaporin TIP1;1 knock-down reduced the sugar translocation in Arabidopsis (Ma et al., 2004). The observed growth reduction in miRNApip1a and miRNApip1b plants might link transmembrane water flow and sucrose translocation *in vitro*.

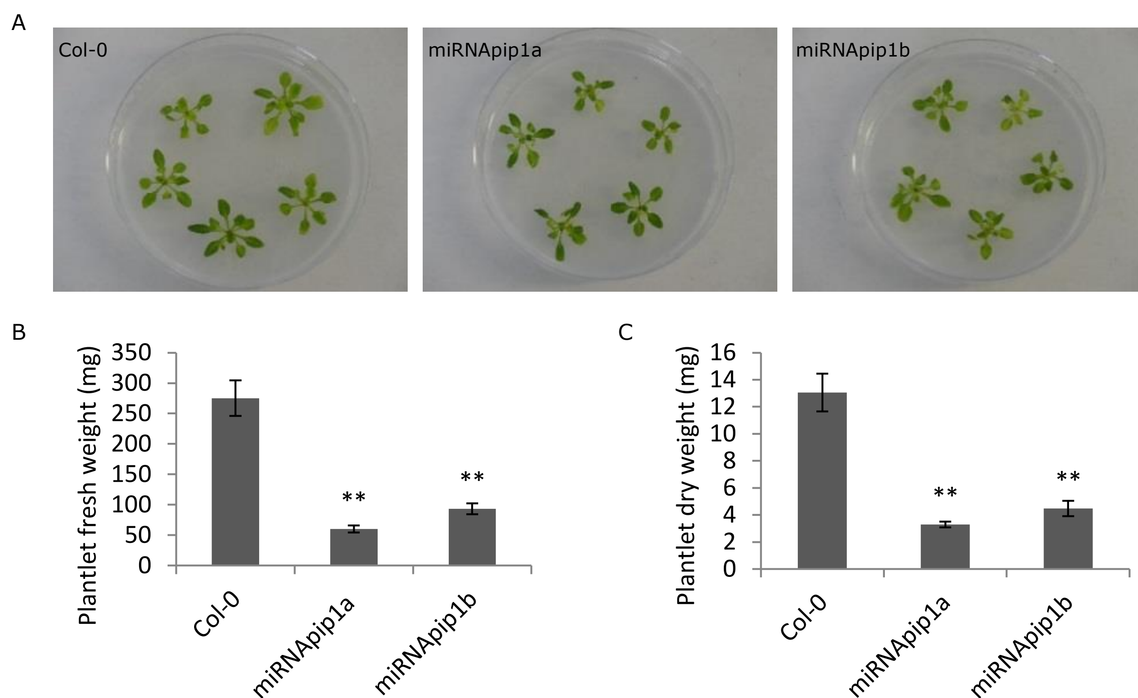

Figure 3. Growth features of miRNApip1a and miRNApip1b plants *in vitro.* A, the morphological appearance of miRNApip1a and miRNApip1b plants compared to Col-0 *in vitro*. B, fresh weights of miRNApip1a and miRNApip1b compared to Col-0 *in vitro* (mean ± SEM, n = 8-10). C, dry weights of miRNApip1a and miRNApip1b compared to Col-0 *in vitro* (mean ± SEM, n = 8-10). Asterisks show significant differences by P < 0.01 (**).

Carbohydrate levels in *in vitro* miRNApip1a and miRNApip1b roots

To explore whether and how PIP1 gene expression, representing water translocation potential, and carbohydrate levels, representing carbohydrate (sucrose) translocation, are linked in *in vitro* Arabidopsis plants we examined the soluble sugar composition extracted from miRNApip1a and miRNApip1b *in vitro* roots. Because of potential catabolization of sucrose from the medium in the roots we looked at multiple hexoses. We found that rhamnose, galactose and glucose were at similar levels in roots of miRNApip1a and miRNApip1b relative to wild type (Figure 4A, 4C & 4D) with e.g. glucose at 14.77 mg/g, 15.19 mg/g, 16.35 mg/g respectively in roots of Col-0, miRNApip1a and miRNApip1b *in vitro*. For arabinose and mannose the levels in roots of miRNApip1b were significantly different from wild type Col-0 while miRNApip1a showed no significant differences (Figure 4B & 4E). The most obvious effect was found in the sucrose levels. The sucrose level was 4.76 mg/g in miRNApip1a and 5.13 mg/g in miRNApip1b, both being significantly higher compared to wild type 4.30 mg/g; this is a 10.70% and 19.30% increase respectively. This overall increase in sucrose concentration in roots in PIP1 silenced lines miRNApip1a and miRNApip1b indicated that PIP1 gene expression might be linked to sucrose accumulation but in a negative correlation as found earlier.

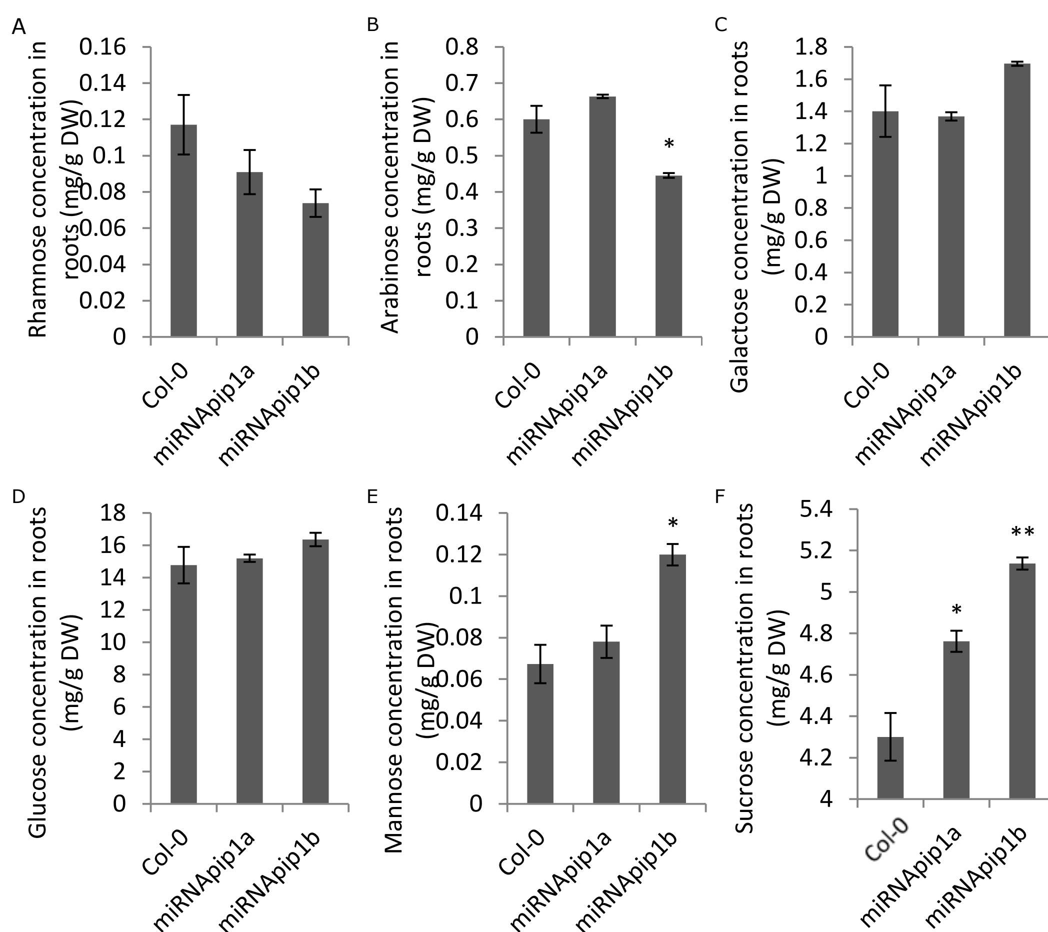

Figure 4. Root carbohydrate contents in Col-0, miRNApip1a and miRNApip1b including rhamnose (4A), arabinose (4B), galactose (4C), glucose (4D), mannose (4E), and sucrose (4F). Results were shown as mean ± SEM, n = 50. Asterisks show significant difference by P < 0.05 (*) and P < 0.01 (**).

Excised root growth in miRNApip1 plants

To reconcile the observed growth reduction in entire PIP silenced plants *in vitro* and the observed higher sucrose accumulation in PIP silenced roots, we studied excised root growth and root biomass development in a root culture system from both miRNApip1a and miRNApip1b and compared it to wild type cultured roots. In root cultures the generated adventitious roots were in direct contact with water and other nutrients, facilitating water and sucrose uptake and translocation (Guillon et al., 2006) while avoiding the influence of leaves and stems. We found that Col-0 formed extensive root clusters in the container, however miRNApip1a and miRNApip1b showed more weak and smaller root systems in culture (Figure 5A, microscopic observations). Illustrative individual root clusters are shown in Figure 5B giving an impression of root sizes of Col-0, miRNApip1a and miRNApip1b. Root length in the wild type Col-0 was generally higher. The fresh weights of PIP1 silenced roots were also determined after two weeks of culture to get more quantitative data: miRNApip1a roots gave a fresh weight of 216.88 mg; miRNApip1b a fresh weight of 187.78 mg, being significantly lower than Col-0 with a fresh weight of 330.96 mg (Figure 5C). Reduced isolated root growth in miRNApip1a and miRNApip1b indicated that water uptake probably was reduced because of lower PIP gene expression and while uptake might be increased leading to higher sucrose levels, sugar assimilation in isolated roots might be impaired, explaining that other hexoses levels were not increased. Sucrose translocation to upper parts of the plant could not take place in these isolated roots. How water translocation and sucrose assimilation are linked is not yet clear and needs further investigations. As transpiration is a major driving force for long-distance translocation of both water and sucrose we performed a transpiration experiment with the PIP silenced plants.

Leaf water retention and PIP gene expression

To establish the role of the aquaporin PIP1 genes in water translocation over longer distances we examined leaf transpiration levels using Col-0 and PIP1 silenced plants *in vitro*. We found the water loss by transpiration was 40.21%, 56.12%, 77.07%, 85.67%, 87.46% and 88.48% at the consecutive time points for the wild type; 35.67%, 55.68%, 77.42%, 84.62%, 86.41% and 87.64% for miRNApip1a; 33.30%, 51.86%, 75.85%, 86.03%, 88.21% and 89.69% for miRNApip1b. The time points were 30 min, 60 min, 120 min, 180 min, 240 min and 300 min after excision respectively (Figure 5D). So, no significant differences were found between wild type and silenced plants in the whole time course. No evidence was found that PIP1 genes were involved in determining leaf water loss as indication for transpiration under these experimental conditions.

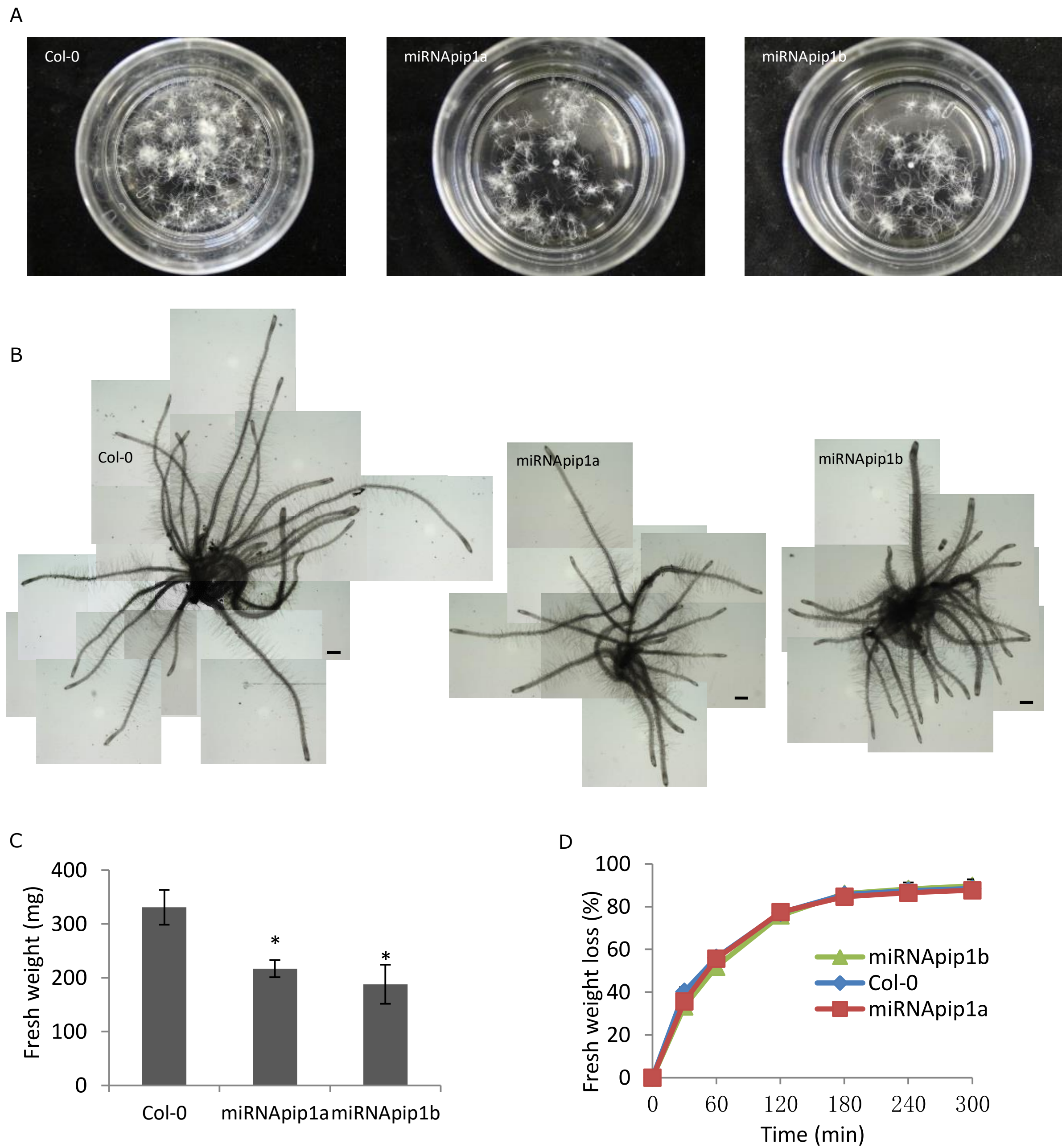

Figure 5. Excised root growth of Col-0, miRNApip1a and miRNApip1b in isolated root cultures and leaf water loss experiment. A, root cultures of Col-0 and lines miRNApip1a and miRNApip1b after 2 weeks. B, root morphology of Col-0, miRNApip1a and miRNApip1b in the root liquid culture system. Scale bars, 500 μm. C, Fresh weight of isolated roots in cultures of Col-0, miRNApip1a and miRNApip1b (mean ± SEM, n = 5-6). D, water loss measured in detached rosette leaves of *in vitro* Col-0, miRNApip1a and miRNApip1b plants at hourly intervals (mean ± SEM, n = 10-15). Asterisks show significant differences by $P < 0.05$ (*).

Discussion

Plant growth *in vitro* is dependent on the control of water homeostasis at the cellular level and on the availability of sufficient nutrients (Creelman et al., 1990; Kozai et al., 1991; Yaseen et al., 2013). The water flux across cellular membranes, important for water homeostasis, is regulated by aquaporins and is involved in diverse processes, such as leaf hydraulic conductivity, seed germination, root water uptake and expansive growth (Chaumont and Tyerman, 2014; Maurel et al., 2015). One might suppose that water flux is closely related to nutrient element translocation, plant growth and yield *in vivo*, but although the function of aquaporins as water channels for transmembrane translocation is well accepted (Maurel et al., 2015; Gambetta et al., 2017), the role that aquaporins play in facilitating nutrient accumulation and mediating plant growth in plant tissue culture is still unclear. To elucidate this role of aquaporins PIP in *in vitro* conditions and in plant growth we investigated the expression of the major PIP gene families, PIP1 and PIP2 and the effect of different sucrose concentrations on expression and we studied the effect of down-regulation of PIP1 genes by microRNAs on plant biomass, carbohydrate accumulation in roots, isolated root growth, and leaf water loss *in vitro*.

The aquaporin gene family consists of multiple subfamilies each with multiple family members. These gene family members share a relatively high level of sequence homology and are redundant in their functions (King and Agre, 1996; Jahn et al., 2004; Nguyen et al., 2013). To reduce the possibility in function substitution or compensation within the PIP1 subfamily, we looked for mutants or lines in which the expression of all PIP1 genes was down-regulated. This was found to be the case in miRNApip1 plants. We confirmed that in the two transgenic lines miRNApip1a and miRNApip1b all members of the PIP1 subfamily were substantially reduced in expression (Figure 2). Moreover, the PIP2 gene expression levels were also partly down-regulated as a side effect in miRNApip1a and miRNApip1b. As a consequence, all differences between wild type, miRNApip1a and miRNApip1b plants in the parameters tested can be attributed to the reduced expressions of the PIP genes (Figure 3).

The effect of sucrose on PIP1 expression *in vitro*

To establish whether there is a link between water flux and nutrient (sucrose) flux or availability we tested the expression of all PIP1 and PIP2 genes in Arabidopsis seedlings grown *in vitro* at sucrose concentrations of 0, 2 and 6%. 0% Sucrose reflecting a low sucrose condition; 2% reflecting a standard tissue culture condition (George, 1993) and 6% reflecting a condition where the available sucrose exceeds the plant's requirement. In case of a positive correlation between the two fluxes one might expect a linear increase in PIP expression or perhaps a peak at 2%. However, we found that expression of all PIP1 genes and some PIP2 genes was reduced by increasing levels of sucrose in the medium with the highest expression at 0% (Figure 1). This suggested a negative correlation and might be an indication that cellular water flux is to some extent inhibited by exogenous sucrose in the medium. Aquaporin expression is regulated by many different and complex factors, at transcriptional, translational and post-transcriptional level (Maurel and Chrispeels, 2001; Hachez et al., 2006). Studies have shown that the abundance of aquaporins is regulated by various hormones or stresses: gibberellins (Lin et al., 2007; Bae et al., 2011; Liang et al., 2013), abscisic acid (Jang et al., 2004; Alexandersson et al., 2005; Zhu et al., 2005), cytokinins and auxins (Lin et al., 2007), and pH (Zhao et al., 2008; Zhang et al., 2013). The difference in regulatory factors

might explain the observed difference in response of the PIP1 and PIP2 subfamilies to sucrose.

To explain the observed sucrose-regulated PIP1 expression pattern it can be envisaged that at 0% all efforts are focussed on translocating the little available sucrose to the sink tissues leading to a high expression of the PIP1 genes. At 2% enough sucrose is available to sustain growth and expression of PIP1 can be reduced as also alternative routes, such as through the apoplast have become active; 6% might be too much, leading to a further reduction in PIP expression as the alternatives will provide sufficient carbohydrates to the sinks. This suggests a negative feedback mechanism, but it is still unknown whether such a particular mechanism exists. Several authors have reported on the perception of excess sugar by hexokinases (HXK), which are sugar-sensing enzymes monitoring glucose levels. Perception of excess sugar by HXK leads to inhibited expression of photosynthetic and aquaporin genes (Xiao et al., 2000; Moore et al., 2003; Kelly et al., 2012). The membrane PIP1 aquaporins themselves might also have a similar sugar perceiving ability and negative feedback mechanism (Kelly et al., 2017). Our results are the first report on a putative negative feedback mechanism between sucrose and PIP expression or water flux under *in vitro* conditions where the carbohydrate source, sucrose, is in the medium and not generated by photosynthesis.

It is important to note that water flux is not only determined by aquaporin gene expression, but that it also largely depends on efficient trafficking of the aquaporin proteins to and embedding in the plasma membrane and on their activity once arrived at the membrane (Kjellbom et al., 1999; Kaldenhoff and Fischer, 2006; Maurel et al., 2008). In some cases, expression levels do not alter that much in response to environmental influences, but, instead, changes at the posttranscriptional level, protein residue phosphorylation or re-localization might occur and result in changes in water flux (Lee and Zwiazek, 2015; Maurel et al., 2015; Wudick et al., 2015). Sucrose has been reported to reduce hydraulic conductivity in roots in a light-dependent manner (Di Pietro et al., 2013) and (Niittylä et al., 2007) found that aquaporin activity in membranes was modulated by sucrose by phosphorylation. Externally added sucrose led to a rapid temporary opening of aquaporin pores followed by a quick subsequent adaptive closing (Niittylä et al., 2007). Phosphorylation and dephosphorylation can influence aquaporin channel gating, pore opening and closing (LUU and Maurel, 2005; Törnroth-Horsefield et al., 2006). Further research is necessary to understand the interaction between PIP1 gene expression, PIP1 activity as reflecting water flux and sucrose availability and translocation.

The effect of lowering PIP1 gene expression on plant growth and root carbohydrate levels in Arabidopsis seedlings *in vitro*

MiRNApip1a and miRNApip1b plants, reduced in PIP1 gene expression, grew weak and stunted *in vitro* probably due to inferior nutrient translocation and cellular water flux. In the wild type Col-0 the contribution of transmembrane water flux by PIP1s in overall water migration for carbohydrate translocation and heterotrophic plant growth was found to be higher (Figure 3). These results suggested a positive correlation between growth and supposedly sucrose translocation and PIP1 expression and water flux. Such a positive correlation was observed earlier *in vivo.* ScPIP1 overexpressed Arabidopsis exhibited longer roots and higher survival rates under stress condition (Wang et al., 2019). Constitutive overexpression of ZmPIP1;1 in Arabidopsis showed enhanced growth

under drought stress condition (Zhou et al., 2018). A soybean line overexpressing PIP showed increased growth in both solution cultures and soil pots (Lu et al., 2018). A tomato line, ectopically expressing the MdPIP1;3, was bigger than the wild type with increased fruit size and fresh weights (Wang et al., 2017).

In roots, two paths exist along which water can be translocated; aquaporins play a key role in cellular water migration (Groszmann et al., 2017), however, an alternative route is provided by the apoplast. In this route, aquaporins (PIPs) do not have a direct function, especially when plants are at a condition in which the transpiration rate is high. Sugar transport is highly likely dominated by the apoplastic pathway, particularly when the stomata are open (Steudle and Peterson, 1998; Steudle, 2001). However, Tournaire-Roux et al. (2003) found evidence to support that most hydraulic water conductivity can be attributed to aquaporin activities (Tournaire-Roux et al., 2003) indicating that the apoplastic path has a modest, or even minor contribution to water transport. Our results support the thesis that the transmembrane, cell-to-cell water migration through aquaporins should not be neglected and might play the predominant role in hydraulic transport and sucrose transport in roots *in vitro*. Hydraulic conductivity in cells as well as in roots is correlated with aquaporins activities (Lee et al., 2005; Ye and Steudle, 2006; Knipfer et al., 2011; Sutka et al., 2011). The reduced heterotrophic growth shown in miRNApip1a and miRNApip1b might be associated with the reduced osmotic hydraulic conductivity mediated by PIP1s and not primarily depended on the remaining, low apoplastic water flow under the *in vitro* conditions.

To be able to further investigate the link *in vitro* between water flux and PIP1 expression to carbohydrate uptake, translocation, assimilation or metabolism we measured in roots the levels of sucrose and several monosaccharides that could be generated by sucrose processing in the PIP-inhibited lines. The content of soluble monosaccharide sugars such as arabinose was lower in miRNApip1b compared to Col-0 while mannose was found to be higher in this line. The other sugars, rhamnose, glucose and galactose, were not significantly changed. In miRNApip1a all monosaccharides were at levels comparable to the wild type Col-0. So, there wasn't a clear link between PIP1 expression and the levels of monosaccharides. However, in both lines, miRNApip1a and miRNApip1b, the sucrose levels in *in vitro* roots were significantly higher compared to Col-0. Sucrose showed a 10.70% and 19.30% increase in miRNApip1a and miRNApip1b respectively relative to wild type. More sucrose accumulating in the roots could come from either enhanced uptake or from impaired upwards-translocation to the sinks. These results confirm that water translocation is not the same as sucrose translocation, although both can follow the apoplastic route as well as a cell-to-cell transmembrane route each having its own translocators. With transmembrane water movement impaired in the miRNApip1 lines, sucrose radial translocation is still functional as evidenced by the high sucrose levels. The fact that in those lines the sucrose levels were even significantly higher than in the control suggested a correlation between PIP expression and sucrose translocation, being a negative one again. In fact sucrose solution transport *in vitro*, no matter through the non-selectivity porous cell wall route or through the trans-membrane route, in which water channel regulate water flow and sucrose transporters mediate sucrose flux, might be related to aquaporins activity directly or indirectly and vice versa (Smith and Griffiths, 1993; Steudle, 2000). The observed impaired upward movement of sucrose in the PIP-inhibited lines, indicated by the reduction in growth and biomass, suggested a further interaction of the two translocation systems, where the altered expression of PIP genes affected driving forces for sucrose translocation (Xu et al., 2018) by influencing osmotic

or hydrostatic hydraulic water movement (Verkman, 2002; Agre, 2006; Hashido et al., 2007; Postaire et al., 2010; del Carmen Martínez-Ballesta et al., 2011). To fully understand the complex interactions between the water and sucrose translocation systems further research is required. We looked at growth of excised roots, so without the influence of upper, aerial parts and at transpiration.

Excised root growth and leaf water retention in miRNApip1 plants

Culturing excised roots in liquid medium showed that adventitious root sizes and root fresh biomass were reduced in PIP-silenced lines (Figure 5A, B & C) indicating that both water and sucrose uptake and translocation along the radial path, either through the apoplast or cell-to-cell, might be impaired in the roots of PIP1 silenced plants. Many research reports have shown the role of aquaporins in root water transport (Quigley et al., 2001; Boursiac et al., 2005; Sakurai et al., 2005; Monneuse et al., 2011). Sucrose translocation in excised roots takes place partly in a radial cell-to-cell pathway (Steudle and Jeschke, 1983; Knipfer and Fricke, 2010b) where the symplast is involved and possibly also aquaporins might play a role, as both systems are linked, as we have shown. The root culture system is a suitable model to study extravascular water flow in which the influence of upper, aerial parts can be excluded. Reduced membrane water flux could be the main reason of the inferior growth *in vitro* shown in isolated silenced roots. Here, the reduced root size is supposed to come from reduced radial loading or uptake of water from the medium and translocation to inner cell layers within the roots rather than from altered transpiration or upwards flow limitation by vascular structures. The reduced fresh weight *in vitro* is supposedly related to a lower hydraulic conductivity caused by silencing the PIP1 genes silencing. Disruption of PIP1;2 led to a noticeably reduced root hydraulic conductivity (20%-30%) (Postaire et al., 2010). However, knockout of AtPIP2;2 showed no alteration in hydrostatic pressure dependent conductivity (Javot et al., 2003).

Transpiration is a major driving force for mass flow of all kinds of nutrients in the xylem from underground parts to the uppermost part of a plant growing *ex vitro* (Medina and Gilbert, 2016). Transpiration might also drive sugar and other nutrients flow in *in vitro* plant tissue culture (see Chapters 2 & 5). Here, we checked the influence of PIP1 aquaporins on long distance water transport, driven by transpiration by measuring the water loss from leaves obtained from *in vitro* grown PIP-silenced and control plants. No difference in transpiration was observed (Figure 5D). This experimental outcome was kind of a surprise as water loss, indicative for stomatal conductance and transpiration capability, has been associated with aquaporin PIP1 expression (Sade et al., 2010). Several reports have shown that aquaporin transcript abundance in guard cells was linked to the control of stomatal movement (Leonhardt et al., 2004; Heinen et al., 2014; Prasch et al., 2015) involved in water outlet. The *in vitro* conditions in our experiment with a long-term high relative air humidity might impair or inhibit the control of transpiration via stomatal pores and cuticular layers (Brainerd and Fuchigami, 1982; Hazarika, 2006; Fanourakis et al., 2011) nullifying the role of PIPs explaining why we did not find any difference in transpiration in our setup. The observed difference in growth and the accumulation of sucrose in roots, therefore, cannot be explained by a difference in transpiration rate between the wild-type control Col-0 and the miRNApip1 lines. Other mechanisms must be involved and elucidation will require further research.

Conclusion

Water translocation and sucrose translocation *in vitro* are linked but not dependent upon each other. Both can use apoplastic and transmembrane routes. The transmembrane route in *in vitro* roots mediated by aquaporin PIP1s is important for growth and sucrose translocation, although the full extent of the interactions and mechanisms are not yet understood. Conditions *in vitro* ensuring high PIP gene expression and optimal PIP activity will favour growth and quality of *in vitro* propagated plants. From our experiments it is clear that overloading the medium with sucrose might not be a good idea, as this inhibited PIP1 and PIP2 gene expression.

Supplemental table list of primers used in the Q-RT-PCR or PCR analysis

name primer	Sequence (in 5'-- 3' order)
UBQ10F	CACACTCCACTTGGTCTTGCGT
UBQ10R	TGGTCTTTCCGGTGAGAGTCTTCA
PIP1;1F	CTGGCCTTGTCCTTAGTTGCTTC
PIP1;1R	ATTCTCCTTTGGAACTTCTTCCTTG
PIP1;2F	TGTGCTTCCGTCGGAATCCAAGGT
PIP1;2R	TCGTGTGAGCGAAAGCTTCCTAGC
PIP1;3F	TCTCAGCCACTCTCATTACCCATTG
PIP1;3R	GGAACTTGTTAGCTCCCACTCGAAC
PIP1;4F	TGGGATGACCATTGGATTTT
PIP1;4R	TCTGGACCGTGGAATCTTTC
PIP1;5F	CCCTATCTTGGCTCCGCTTCCAAT
PIP1;5R	TGTAGATGATGGCAGCTCCGAGAC
PIP2;1F	CGTAGATTGCGGCGGAGTTGGAAT
PIP2;1R	CGCTGGGTTAATGTGACCACCAGA
PIP2;2F	CAGAAGTGCAGCCAACGTTTGAG
PIP2;2R	CATTGGCATTGGCACATATCAAAG
PIP2;3F	TTCGACGCAGAGGAGCTTACCA
PIP2;3R	TACGAACTCGGCGATGACTGCT
PIP2;4F	CCGATGGCTACAACAAAGGT
PIP2;4R	CACGTGAGAGTCACGAGCAT
PIP2;5F	TGGTGGGCATATTAATCCGGCAGT
PIP2;5R	TGACCAAAGCCACACCACAAATGG
PIP2;6F	CGGCATCTCTGGTGGACACATCAA
PIP2;6R	AACCAACTCCACAAGTGGCTCCGA
PIP2;7F	GCTCCTCTTCTCGACATGGGTGAG
PIP2;7R	CTTGTGGCCGATGACAGTAGCGAC
PIP2;8F	TCCCGGTTTTGGCTCCGTTACCTA
PIP2;8R	TCCAATGGTCGTCCCAAGCTTTCT
Mir164F	CTCTCACCACAAATGCGTGT
Mir164R	ACACTTGAACCCTCGTCGTC

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CHAPTER 5

Transpiration-driven plant growth in apple *in vitro* micropropagation

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Abstract

Micropropagation enables vegetative production of large numbers of plants in a short period of time. One of several multiplication methods is the induced formation of adventitious buds and shoots; another is the forced outgrowth of axillary bud meristems. For growth, the conditions *in vitro* are far from 'normal' and mechanisms that are regulating growth are still largely unknown. A major question is how medium components are translocated to the areas of growth. In general most translocation occurs through xylem and phloem and is driven by transpiration. It has been shown already that transpiration indeed occurs in tissue cultured plants. It is assumed that poor transpiration might be one of the reasons of poor performance in tissue culture. In our experiments, shoot cultures of *Malus domestica* cv. 'Gala' were used to test the relationship between transpiration and growth. The hypothesis is that an increased transpiration potential might lead to improved growth of plants.

A number of things was attempted to show that transpiration did occur *in vitro* such as containers with different filters fitted in the lids and dye migration in cultured plantlets. We showed that the rate of transpiration was depending on several environmental and physiological factors including the humidity level, stomatal aperture, and cuticle status. High gas replacement filters stimulated the increase in plant weight, both fresh and dry weight, as shown by 'Gala' shoots growing in culture with lid filters varying in gas exchange rates. As a measure for transpiration the use of a dye, fuchsine in experiments on shoots with and without leaves clearly proved that transpiration did occur. Plants grown in reduced relative humidity by application of a small vial filled with a highly concentrated potassium chloride solution (hygroscopic) in the container exhibited an increased transpiration and higher dry weight. Opening the stomata also increased transpiration and biomass accumulation: apple shoots in medium treated with δ-aminolevulinic acid displayed enhanced stomatal aperture sizes and water loss, and also showed an increase in biomass compared to the controls. In addition, we monitored the effect of adding an herbicide, metolachlor, which is supposed to inhibit cuticle formation and should lead to enhanced cuticular transpiration. However, the metolachlor treated shoots did not exhibit enhanced fresh and dry weight or higher water loss. Our results demonstrated the potential of modulating transpiration to optimize biomass accumulation *in vitro*. These results in apple confirmed our earlier results obtained with *Arabidopsis thaliana in vitro* grown seedlings. This study provides strong evidence that water translocation and transpiration have a strong synergistic influence on boosting the plant growth *in vitro*. Similar techniques to enhance transpiration might also be applied to other species or cultivars growing poorly in tissue culture.

Keywords: δ-aminolevulinic acid, plant growth, transpiration, stomata, cuticle, *Malus domestica* 'Gala', micropropagation

Introduction

Micropropagation of apple, a woody plant, provides a fast and reliable method producing many genetically uniform plants. Apple *(Malus × domestica* Borkh.) is one of the most widely cultivated fruit crops after citrus, grape, and banana (Janick, 2005) and has significant economic importance (Hummer and Janick, 2009; Gross et al., 2014). Apple as a crop has received increasing attention from both the scientific community as well as from growers as indicated by the increasing number of articles not only in fundamental studies, but also on practical applications (Eccher et al., 2014). The traditional apple propagation methods are vegetative transplanting, cutting, grafting and budding. The conventional methods fail to ensure virus-free planting material and moreover, they are featured with low propagation rates and high costs. However, tissue culture propagation may overcome some of these limitations as in micropropagation environmental factors can be controlled and subculture can be done continuously (Idowu et al., 2009). Current micropropagation technology provides a method allowing the production of a large quantity of genetically identical and disease-free plants all year round (Zimmerman, 1981).

To optimize plant growth *in vitro,* the factors and conditions that might allow higher levels of nutrient acquisition are the focus of tissue culture research. The observation that physiological features and growth speed of plants in tissue culture are very different from those of plants in the field, might be attributed to the *in vitro* culture conditions: tissue or organ originated explants are cultivated in an aseptic, sealed environment with low gas exchange. A combination of external factors in this environment inhibit optimal growth in containers, for instance, limited air ventilation, high relative humidity (RH) or low vapour pressure deficit (VPD) in the headspace, low light quality and varying CO_2 content in the vessels in light and dark periods (Proft et al., 1985; Predieri et al., 1990; Dobránszki and da Silva, 2010). In this culture environment the nutrient source for plants mainly stems from the artificial culture medium, conducive for growth and multiplication, mostly consisting of carbohydrates, vitamins, plant hormones and mineral ions (Murashige and Skoog, 1962). It is an option to enhance nutrient upward translocation for improving sustainable *in vitro* growth.

A correlation between transpiration-induced water flow and plant growth has not been established yet in plant tissue culture. The transportation of supplied nutrients is essential for growth in tissue culture in such a high humidity aerial environment. Medium nutrients are dissolved in and transported together with water in a flow ultimately leading to biomass accumulation or an increase in growth (Kozai et al., 1991; Kozai, 2010). It has been proposed that decreased humidity in jars could promote micropropagated plant transpiration and therefore could enhance nutrient absorption associated with higher numbers of good quality plants (Zimmerman, 1994; Kozai et al., 1995; Aitken-Christie et al., 2013). Elevated transpiration is shown to be responsible for a stimulated upward translocation of water and dissolved carbohydrates (Roberts et al., 1994).

Stomata serving as valves and the cuticle acting as a barrier-layer between epidermal cells and the environment, both enable plants to fine regulate water transpiration levels and water homeostasis independent from roots. Cuticular transpiration contributes less than 10% to the plant's transpiration in total *ex vitro* (Kerstiens, 1996, 2006). Still, cuticular water permeability can determine transpiration and can play an essential role in minimizing water loss. Reducing or damaging the leaf epicuticular wax layer might lead

to an increase in transpiration *ex vitro* (Sutter and Langhans, 1982; Preece and Sutter, 1991; Roberts et al., 1994) and *in vitro* (Chapter 2). Apart from cuticular transpiration, stomatal pores are dynamically adjusted in size thereby regulating the rate of transpiration as well (Willmer and Fricker, 1996). Active and open stomata are associated with transpiration *ex vitro*, leading to substantial nutrient transport and water flow upwards. A positive correlation has been established in Chapter 2 between stomata and transpiration *in vitro*.

In the previous chapters we studied the effects of transpiration and of sucrose and water translocation in the model plant, *Arabidopsis thaliana.* To see to what extent the results obtained there could be extrapolated to other, commercially more important crops, we set up research in apple *in vitro* propagated shoots, a system also devoid of roots. Here, we investigated whether a correlation existed between leaf transpiration and plant growth *in vitro* aiming to optimize plant growth and biomass accumulation. We examined the influence of microbox filters varying in gas replacement rates on shoot growth. Apart from that it was tested whether transpiration occurred *in vitro* and whether nutrient translocation was influenced by leaf transpiration. Furthermore, to understand the relationship between humidity and shoot growth we regulated headspace humidity in vessels by saturated potassium chloride. We examined if the reduced humidity in jars had a beneficial effect on plant transpiration and plant biomass accumulation. In addition, we attempted to increase transpiration through the cuticle on the epidermal surface and through stomatal pores. We investigated whether opening stomata of leaves may stimulate transpiration and biomass accumulation. Moreover, we studied the effect of adding an herbicide, metolachlor, which should inhibit cuticle formation, on water loss and biomass accumulation *in vitro*. In summary, we established that it is possible to increase growth of micropropagated shoots *in vitro* through manipulation of transpiration.

Materials and Methods

Plant materials

Shoot segments of *Malus domestica* apple cultivar 'Gala' were obtained from the field and transferred to tissue culture. To sterilize the material the shoot fragments were washed under running water and subsequently submerged in 3% (w/v) hypochlorite (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 30% (w/v) Tween 20 (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 5 minutes and finally steeped in 70% (v/v) alcohol (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 20 minutes. The shoots were then rinsed 3 times in sterile de-ionized water. These shoots were placed in plastic jars supplemented with autoclave-sterilized (121°C for 20 minutes) propagation medium (pH 5.7-5.8) which consisted of full strength MS salts and vitamins (Duchefa, Haarlem, The Netherlands)(Murashige and Skoog, 1962), 2% (w/v) sucrose (Duchefa, Haarlem, The Netherlands), 266.39 μM Fe-EDDHA (Duchefa, Haarlem, The Netherlands), 9 g/L Daishin agar (Duchefa, Haarlem, The Netherlands), and 3.11 μM 6-bezylaminopurine (BAP)(Duchefa, Haarlem, The Netherlands). These shoots were subcultured every 6 weeks on fresh culture medium in a climate chamber (Conviron BDR16)(Conviron Germany GmbH, Berlin, Germany) with a temperature at 24°C and light intensity 50 μmol m^{-2} sec^{-1} (Philips Master TL-D 36)(Philips, Poland) in 16 h light and 8 h darkness periodic rhythm.

Effect of gas exchange rate on shoot growth

Shoots, carefully-selected for a similar diameter, were divided into fragments of 2-centimeter length and were cultured in a microbox (base 125 mm * 65 mm, height 80 mm and volume 540 ml; Eco2 NV, Geraardsbergen, Belgium) in a climate chamber for 6 weeks. These microboxes were transparent polypropylene boxes closed with a polypropylene cover which contained a filter made of a double row of hydrophobic materials. Of the boxes, 4 different filter types were utilized: white, yellow, red and green, each of which had a unique gas exchange rate being 7.44 gas replacement (GR)/day, 9.87 GR/day, 10.83 GR/day and 62.87 GR/day respectively. 10 Shoots were cultured per container for 6 weeks and subsequently fresh weight and dry weight were determined using a XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands).

Water flow visualization by acid fuchsine in micropropagated shoots

6-Weeks-old micropropagated apple shoots were collected and individual shoots were isolated. To obtain defoliated shoots all leaves were removed using forceps (Medicon, Tuttlingen, Germany) and scalpel (Medicon, Tuttlingen, Germany) while intact shoots for comparison were left untouched. Subsequently, these two types of shoots were inserted in propagation medium supplemented with 1% (w/v) acid fuchsine (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Defoliated shoots and intact shoots were cultured in the same climate chamber for a few days and then stems were manually cut into small sections of approximately 0.5-1 mm thick, followed by stem section observation under Zeiss Stereo Discovery.V8 Zoom Stereo Microscope (Carl Zeiss Microscopy, White Plains, United States) or under an Axiophot light microscope (Carl Zeiss Microscopy, White Plains, United States) equipped with AxioCam ERc5S digital camera (Carl Zeiss Microscopy, White Plains, United States).

Humidity regulation in culture and effect on transpiration and growth

Micropropagated shoots were inserted in tubes containing 5 ml propagation medium and then the media were subsequently covered with 1 ml paraffin oil (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) to block evaporation from the medium surface. One vial containing 4 ml saturated potassium chloride solution and 2 g KCl tablets (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was put in the plastic culture jars to decrease humidity. Each treatment had 28 shoot individuals in jars with either no paraffin oil covering the medium surface, or with paraffin oil; and with either no potassium chloride added or saturated potassium chloride added. Apple shoots were cultured in the jars for 4 weeks under described treatments of paraffin oil and potassium chloride. Transpiration and dry weight were measured at the end of week 4. The whole tube containing medium and shoot was measured twice by XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands) at the first day and third day in three days period after week 4. The weight difference was taken as the amount of water transpired and was divided by the integrated leaf area of the whole shoot which was counted by image analysis software Image–J-1.4 (United States National Institutes of Health and the Laboratory for Optical and Computational Instrumentation in University of Wisconsin, USA). For the latter, all leaves were collected and fixed flat on paper, scanned by image scanner, next to area calculation. The shoot dry weights were measured after drying the apple shoots in an oven (Omnilabo International B.V., Breda, The Netherlands) at 70 °C overnight.

Stomatal aperture and density measurement

The stomata on the abaxial side of leaves were visualized by using dental resin impressions (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands)(Kagan et al., 1992; Berger and Altmann, 2000). The resin was prepared by mixing equal amounts of catalyst and base resulting in silicone elastomer polyvinylsiloxane. Subsequently, the abaxial side of leaves was carefully pressed onto the silicone and peeled off after the silicone had dried. Transparent nail polish (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was applied to the resin imprints (von Groll et al., 2002). Dry nail polish film was separated and mounted on slides for further microscopical inspection. Imprints were observed under bright field (Axiophot light microscope) and micrographs were taken using an AxionCam ERc5S digital camera attached to the microscope (Carl Zeiss Microscopy, White Plains, United States). The stomatal characteristics on these dry nail polish film imprints were used to determine stomatal density and aperture using image analysis software Carl Zeiss Zen 2.3 lite (Carl Zeiss Microscopy, White Plains, United States). Stomatal density was measured in fields of view by 0.2 mm X 0.2 mm per leave. Aperture size was examined in 20 to 30 guard cell pairs in microphotographs by 0.2 mm X 0.2 mm. Stomatal opening was forced by administering 29 μM δ-aminolevulinic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and its effect was monitored. The third and fourth fully developed leaves were collected from 5 weeks-old apple shoots grown in propagation medium supplemented with 29 μM δ-aminolevulinic acid in a climate chamber. The stomatal aperture size and number were examined as described above from around ten shoots per treatment.

Water loss measurement

5-Weeks-old shoots were selected for being similar in size and shoots were subsequently excised and examined in open Petri dishes (Greiner Bio-One B.V., Alphen aan den Rijn,

The Netherlands) on the work bench at room temperature during the measurement. The Petri dishes containing plant tissues were weighed on the XPE105 Analytical Balance (Mettler Toledo B.V., Tiel, The Netherlands) at different time points. The rate of water loss indicative for water transpiration was determined as ratio of weight lost at each time point to the initial fresh weight (Gonzalez-Guzman et al., 2012).

Shoot growth influenced by cuticle removal

Malus domestica 'Gala' shoots were collected after 5 weeks of growth on propagation medium supplemented with 30 μM metolachlor (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). The water loss and biomass weight were determined as described earlier. In addition, *Malus domestica* 'Gala' shoots were grown in tubes (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) with or without metolachlor and after 5 weeks of growth they were collected for leaf treatments. The adaxial side of leaves was scrubbed using cotton sticks, which were dipped with water, chloroform (Biosolve BV, Valkenswaard, The Netherlands), hexane (Biosolve BV, Valkenswaard, The Netherlands), diethyl ether (Biosolve BV, Valkenswaard, The Netherlands) separately, for 10 seconds respectively. Tubes which contained treated shoots were transferred back to a container for further investigation. The shoot growth was monitored and photographed within one week after treatments for comparison.

Statistical analysis

All statistical analyses were conducted with GraphPad Prism (GraphPad Software, San Diego, USA) and OriginPro software (Originlab, Wellesley Hills, USA). Significance was determined by one-way analysis of variance (ANOVA) Bonferroni-Holm adjustment test or Student's *t*-tests. Asterisks above each bar represent statistically significant differences (*, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$). Different letters above each bar represent statistically significant differences ($P < 0.05$).

Results

The effect of gas exchange rate on *in vitro* growth

To investigate the role of the gas exchange rate on shoot growth in *in vitro* containers we obtained transparent polypropylene containers with covers with 4 different filter types. The amount of potential gas replacement per time unit as provided by the manufacturer (Eco2 NV) is shown in Figure 1A: green filter showed the maximum gas replacement rate at 62.87 GR/day, next to the red filter at 10.83 GR/day, yellow filter at 9.87 GR/day, and white the lowest at 7.44 GR/day. Apple shoots were cultured in these containers and showed differences in fresh weight and dry weight (Figure 1B and Figure 1C): green filter containers had the highest amount of biomass, 292.14 mg fresh weight and 49.61 mg dry weight while white filter boxes had the lowest amount of biomass, 169.61 mg fresh weight and 27.15 mg dry weight. Red filter boxes and yellow filter boxes had intermediate amounts of fresh biomass, 239.87 mg and 224.42 mg, respectively; and of dry weight, 38.72 mg and 37.23 mg, respectively. The green filter vessels had significantly more dry weight than the other three filter vessels, and its fresh weight was the highest among these filter type vessels. The filter parameters of the covers affect gas exchange rate and had a pronounced effect on fresh and dry biomass, probably through determining the water status in the vessels and ultimately in the plants. The growth morphology of these micropropagated shoots in the 4 filter type containers is displayed in Figure 1D. It was found that white filter containers resulted in small sized shoots; yellow and red filter containers had intermediate sized shoots and green filter containers yielded the largest shoots, so size matched to filter gas replacement rates in these containers.

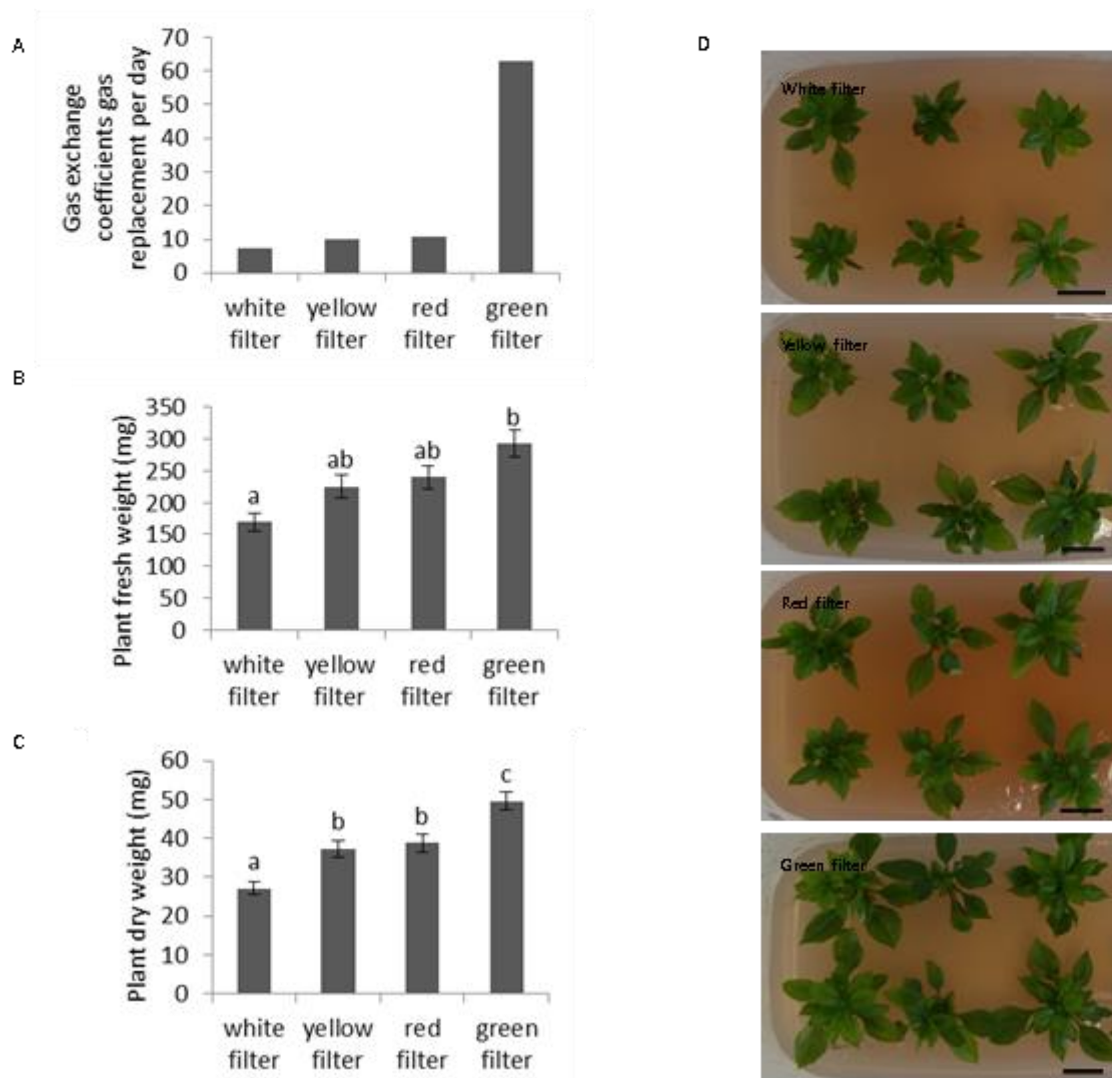

Figure 1. Effects of different gas exchange filters on *Malus domestica* 'Gala' shoots growth *in vitro*. (A) Gas exchange coefficients (GR) per day in 4 type filter containers. (B) The fresh weight of each apple shoot after 6 weeks growth in 4 types of filter containers (mean ± SEM). Each filter treatment had 20 individuals measured from two vessels. (C) The dry weight of each apple shoot after 6 weeks growth in 4 types of filter containers (mean ± SEM). Each filter treatment had 20 individuals measured from two vessels. (D) Representative photographs of 6-weeks-old shoots in containers with the 4 types of filters. Bars, 10 mm. Different letters above each bar represent statistically significant differences ($P < 0.05$).

The effect of leaf transpiration on medium dye translocation

We investigated whether leaf transpiration occurred *in vitro* and whether leaf transpiration was associated with nutrient transportation *in vitro*. The fully intact shoots, so with leaves, and defoliated shoots were grown in media which contained acid fuchsine as the proxy of a nutrient to be taken up. The intact shoots displayed faster fuchsine transport in their stems compared to defoliated shoots as shown in Figure 2A. The stems were still green in defoliated shoots, however, stems and leaves were stained pink or red in intact shoots (Figure 2A), indicating more acid fuchsine absorption and translocation in intact shoots. The schematic drawing (Figure 2B) represents callus-based shoots of which the markings, section 1 to section 6, were the node points where stem specimens were collected for investigation. Stems were cut into sections positioned low, section 1, to higher up the stem of the shoot, section 6. The staining intensity in Figures 2C & 2D present the amount of acid fuchsine and water translocated. Stem cross sections from intact and defoliated plants were examined as isolated from their various locations at indicated time points (Figure 2C and 2D). Removing leaves considerably diminished staining (Figure 2C)—low locations were stained but high locations were not which was different in intact shoots, where all sections were stained on days 3, 4 and 5 (Figure 2D). The acid fuchsine translocation pattern in the two different types of shoots indicated that the presence of leaves was of great influence on water translocation, most likely by transpiration *in vitro*, and that water loss through leaves (transpiration) determined the flow of dissolved elements in plants *in vitro*.

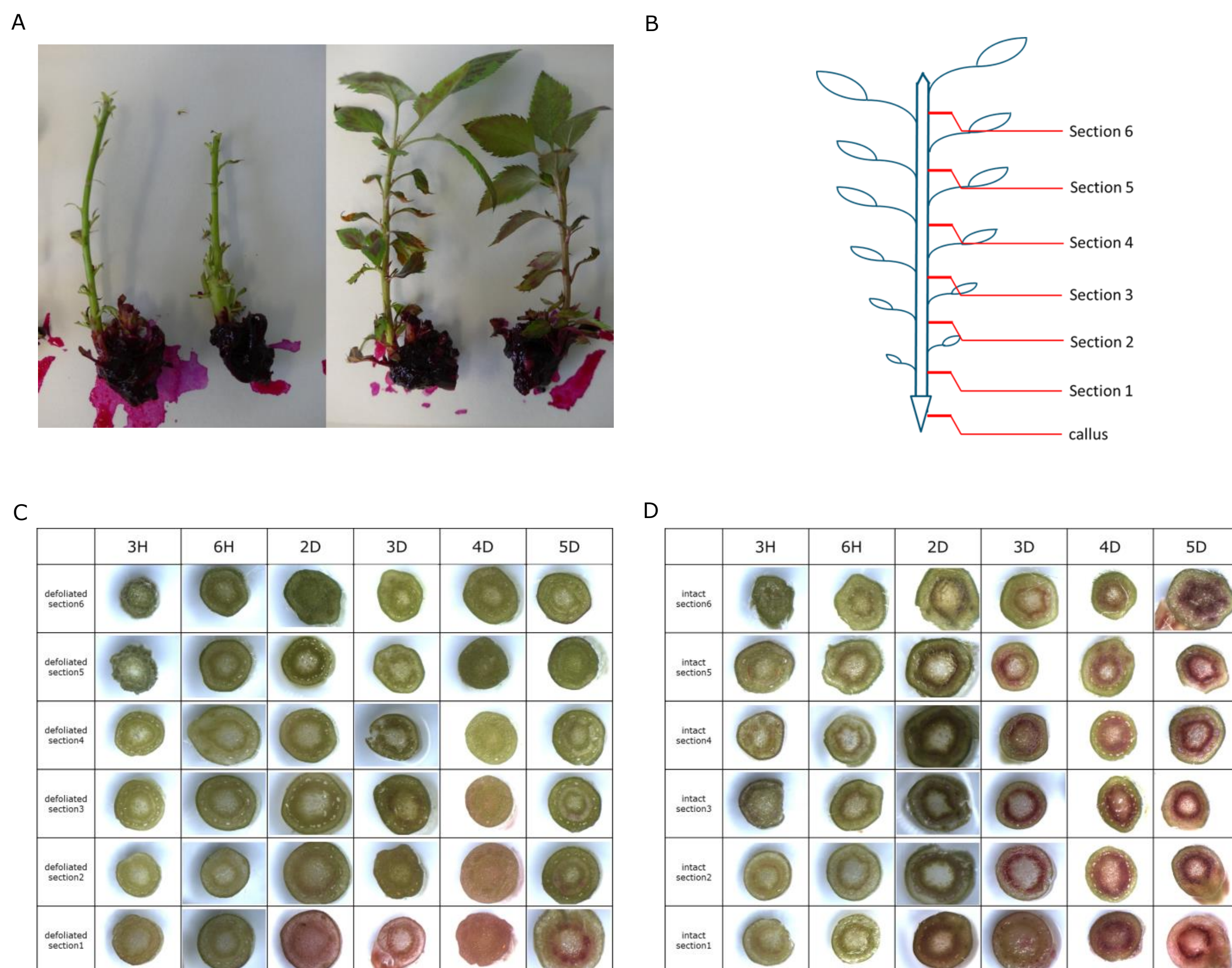

Figure 2. Effects of leaf transpiration on the flow of acid fuchsine solution in apple shoots. (A) The morphology of defoliated shoots (left) and intact shoots (right) grown in propagation medium supplemented with 1% (w/v) acid fuchsine for 2 days. (B) A schematic view of propagated shoots depicting the various locations of stem sections for microscopical analysis. (C) Various stem sections stained by acid fuchsine, from low position (bottom) to high position (top), in defoliated shoots at different time periods, 3H, 6H, 2D, 3D, 4D and 5D. H represents hour; D represents day. (D) Various stem sections stained by acid fuchsine in intact shoots at different periods of time. 10 Individual shoots were used per treatment.

The effect of altering relative humidity on transpiration and growth

To examine the possible correlation between the humidity inside the growth containers and the transpiration and growth of *in vitro* cultured shoots a hygroscopic saturated potassium chloride (KCl) solution was used to absorb moisture from the headspace in jars. Transpiration and biomass weight were measured in these treatments after a culture period of four weeks. To prevent interference from evaporation directly from the medium paraffin oil was used to cover the gellified surface. Approximately 30 shoots were grown under each of three conditions: no paraffin oil covering the medium surface and no potassium chloride absorbing moisture (-Paraffin oil, -KCl); paraffin oil covering but no potassium chloride (+Paraffin oil, -KCl); paraffin oil covering and saturated potassium chloride added (+Paraffin oil, +KCl)(Figure 3A). Saturated potassium chloride was able to absorb the container's headspace moisture in a consistent way, leading to a higher transpiration rate compared to no potassium chloride added (Figure 3B). Water loss observed in the treatment without paraffin oil added probably arose by two mechanisms, medium evaporation and leaf transpiration. As expected, the dry biomass weight increase in the saturated potassium chloride treatment was the highest (Figure 3C). Apparently, potassium chloride in vessels could reduce water vapour pressure and consequently regulate water loss/transpiration and dry biomass accumulation of micropropagated shoots.

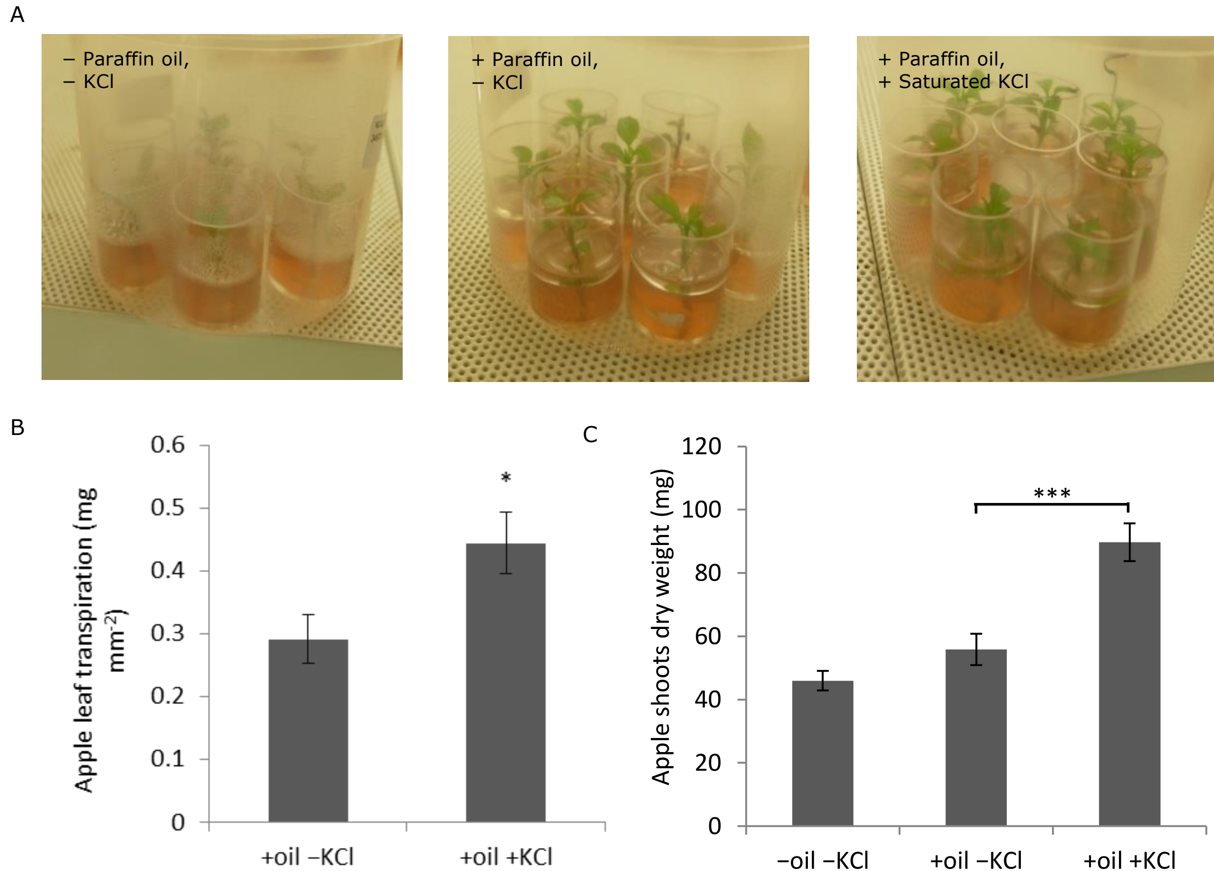

Figure 3. Effects of potassium chloride on transpiration and dry weight of apple shoots. (A) Apple shoots in jars under treatments of paraffin oil covering medium surface and addition of vials of potassium chloride. "−paraffin oil" means no paraffin oil covering the medium surface while "+paraffin oil" means paraffin oil covering the surface. "−KCl" means no vial of potassium chloride added while "+KCl" means saturated potassium chloride added. (B) Transpiration per leaf area of shoots in jars with paraffin oil and with or without potassium chloride regulating internal humidity (mean ± SEM). Values represent 10 measurements. (C) Shoots' dry weight after 4 weeks of growth as influenced by paraffin oil and potassium chloride (mean ± SEM). Values represent around 30 measurements. Asterisk above the column represents a significant difference ($P < 0.05$). Asterisks above the column represents a significant difference ($P < 0.001$)

The effect of δ-aminolevulinic acid on stomata opening *in vitro*

Now that we confirmed that transpiration plays an important role in *in vitro* growth, we focused on studying first the contribution of transpiration through the stomata to growth and whether *in vitro* shoot stomata might be manipulated in culture for growth enhancement. For this, we evaluated the effect of δ-aminolevulinic acid (ALA) on guard cell behaviour in stomata and on the biomass of *in vitro* grown shoots. Exogenously applied δ-aminolevulinic acid has been reported to increase the aperture width of stomata, the total pore area of stomata, and seedling biomass when plants were grown either in soil medium or on paper towels *ex vitro* (Zhao et al., 2015; Anwar et al., 2018; Han et al., 2018). The stomatal density and aperture size were monitored using 29 μM ALA in treatments (Figure 4A). Stomatal density was not significantly changed in ALA supplemented medium compared to the control (Figure 4B). On the other hand, stomatal aperture size was significantly increased upon 29 μM ALA treatment, 13.19 μm, compared to the control, 7.90 μm (Figure 4B). We verified that ALA induced larger stomatal opening *in vitro*.

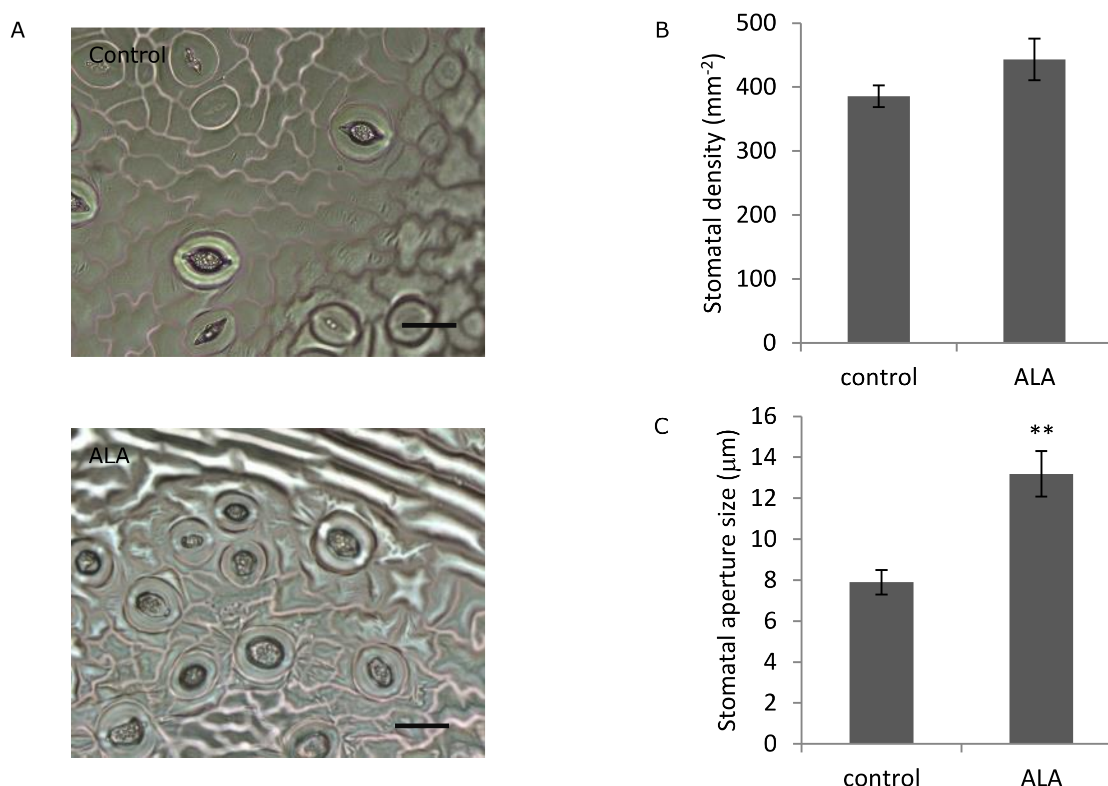

Figure 4. Stomatal density and aperture size of stomata on the abaxial surface of leaves of apple shoots as affected by treatment with δ-aminolevulinic acid (ALA). (A) Stomatal photographs of leaves originated from apple shoots by ALA treatment at 29 μM compared to control no ALA. Scale bars represent 50 μm. (B) Stomatal density of leaves from plants treated with or without ALA in number per square mm (mean ± SEM). (C) Stomatal aperture size as measured after treatment of ALA and treatment without ALA (mean ± SEM). Values represent around 30 measurements. Asterisks above the column represent a significant difference ($P < 0.01$).

The effect of exogenous δ-aminolevulinic acid on stomatal transpiration and growth

Transpiration is an essential driving force for the upward movement of water as well as of nutrients through the vascular system and therefore we studied whether stomatal transpiration could be increased by increasing the total stomatal pore aperture size and examined its effect on water loss and biomass in ALA treated shoots *in vitro*. 29 μM ALA was chosen as the optimal concentration in the following experiments. Water loss was investigated in leaves of ALA treated shoots in a period of 3 hours after excision (Figure 5A). The difference of water loss between the control and ALA treated shoots indicated that the ALA-induced in stomatal opening resulted in an increase in transpiration *in vitro* (Figure 5A). For monitoring the effects on shoot growth and biomass, fresh and dry weights were compared between 'Gala' control (-ALA) shoots and 'Gala' shoots grown in ALA-supplemented medium (Figure 5B). Exogenous ALA application at 29 μM increased fresh biomass, 969.50 mg compared to 876.55 mg fresh weight in control shoots, a 10.61% increase, although no increase of dry weight was found (Figure 5B), demonstrating that ALA only had a beneficial effect on fresh biomass production *in vitro*. Representative photographic images showed that ALA application at 29 μM accelerated shoot growth in culture (Figure 5C). It seems ALA might be an option to enhance shoot growth through promoting transpiration *in vitro*.

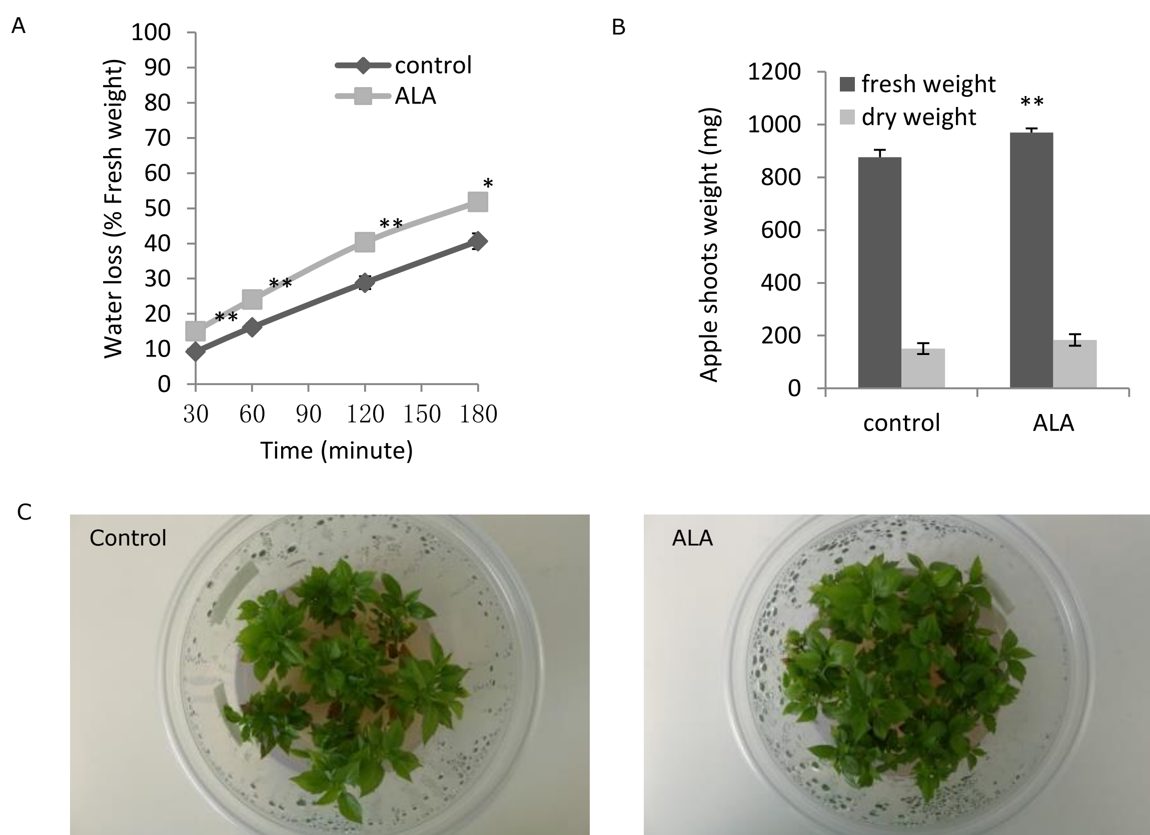

Figure 5. The effects of exogenously applied ALA on leaf water loss and shoot biomass accumulation in *in vitro* cultured *Malus domestica* 'Gala'. (A) Leaf water loss as a percentage of the fresh weight at indicated time points (mean ± SEM). (B) Fresh weight and dry weight in control and in shoots treated with ALA (mean ± SEM). (C) Representative photographic images of 5-weeks-old shoots, control and ALA treated. Values represent 20-30 measurements. The ALA concentration used was 29 μM in all experiments. Asterisks denote statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) relative to the control.

Attempts to increase cuticle transpiration and shoots growth

In addition to stomatal transpiration *in vitro* plants might lose water through the cuticle as well and we found that in *Arabidopsis thaliana in vitro* seedlings cuticular transpiration existed and contributed significantly to nutrient assimilation and growth (Chapter 2). To further assess its role in water or nutrient flow in apple shoots *in vitro* the effect of metolachlor on water loss and shoot growth were examined. The herbicide metolachlor is utilized in agriculture and inhibits the formation of main wax components related to the cuticle by limiting the synthesis of very-long-chain fatty acid (VLCFA). We applied metolachlor for stimulating cuticle transpiration and monitoring growth by limiting cuticle formation. However, the levels of water loss between control and metolachlor treated shoots showed no significant difference (Figure 6A). Also, no difference was seen in fresh and dry weights comparing control with metolachlor treated shoots (Figure 6B). It was evident that metolachlor treatment by incorporating it in the medium in our hands was not effective in increasing cuticular transpiration and biomass production in *in vitro* grown apple shoots. In another approach, organic solvents were used to remove the surface cuticle. Cotton sticks were used to rub leaves on the adaxial side after the cottons were submerged in chemical solvents, such as chloroform (Ⅱ), hexane (Ⅲ), diethyl ether (Ⅳ), in an attempt to remove the surface cuticle (Figure 6C). The leaf phenotype was photographed two days after the treatments, however it was found this method severely impacted the leaves: parts of the leaf area wilted and became necrotic indicating that the method used was not suitable for growth enhancement of *in vitro* shoots. In these ways, so far, the contribution of cuticle transpiration to the *in vitro* of apple shoots could not be established.

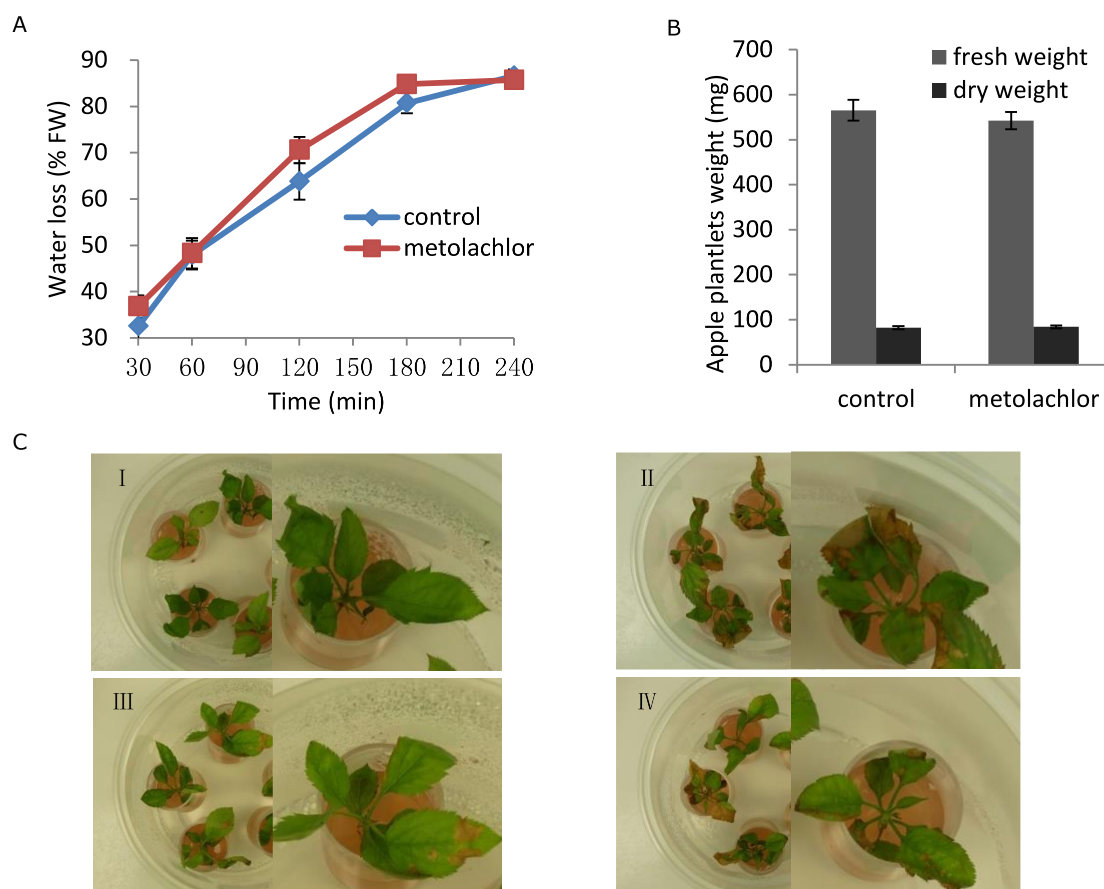

Figure 6. Effects of cuticle removal on transpiration and growth *in vitro*. (A) Leaf water loss in a time course at indicated time points of apple shoots grown in medium without (control) or with 30 μM metolachlor (mean ± SEM, n > 20). (B) Apple shoots fresh weight and dry weight after 5 weeks of growth on 30 μM metolachlor medium relative to the control (mean ± SEM, n > 20). (C) Effects of removing the cuticle on growth in an assay using cotton sticks to rub leaves on the adaxial side after the cottons were submerged in chemical solvents, such as (Ⅰ) water, (Ⅱ) chloroform, (Ⅲ) hexane, (Ⅳ) diethyl ether respectively.

Discussion

For *in vitro* cultured plants, water moves starting from the gellified medium, enters the roots, goes through the xylem, and evaporates from leaves into the headspace atmosphere. Dissolved nutrients might also go along in this so-called medium-plant-atmosphere-continuum (MPAC). For such nutrient translocation and water upward flow water potential differences between medium and cultured plants might be the driving force. It is important to note that the efficiency of upward translocation of water, minerals and carbohydrates is a vital determinant for growth *in vitro*. Nonetheless, scanty knowledge is available on how medium nutrients, sucrose, minerals and water are translocated internally in cultured shoots. It has not been well investigated to what extent transpiration occurs in culture vessels and how transpiration contributes to the translocation of supplied nutrients under high humidity. We initiated experiments to study this in order to understand what determines shoots growth *in vitro* and whether it is possible to optimize shoots growth by stimulating transpiration.

The influence of gas exchange rates on *in vitro* growth

To understand the influence of gas exchange rates on shoot growth we utilized various lid filters differing in rates and examined shoot biomass increases. Based on information from the manufacturer, the green filter had the highest gas exchange coefficient value. The average fresh weight and dry weight of shoots were obtained in containers with green filters compared to all other vessel lids (Figure 1). These results clearly showed that a higher gas exchange rate was beneficial for *Malus domestica* ‘Gala’ growth in tissue culture. The low gas exchange in almost completely sealed containers is held responsible for the low water retention capacity and physiological problems related to the *in vitro* culture (Debergh and Maene, 1984). Several articles have suggested that poor gas exchange and restricted aeration greatly influenced growth characteristics *in vitro*; marginal transpiration and minimal transpiration-driven nutrient migration in sealed container *in vitro* were suggested as causes for this (Blazková et al., 1988; Buddendorf-Joosten and Woltering, 1994; Majada et al., 1998). In our studies we clearly demonstrated the relation between gas exchange and *in vitro* growth, not just for transpiration but to sustain healthy growth, and internal gases in the vessel, such as ethylene produced by the plants or CO_2 necessary for photosynthesis, must be delivered or exchanged as efficient as possible. Tubes with higher gas exchange rates have been used to culture species such as carnation (Kozai and Iwanami, 1988).

The increased air movement probably facilitates both carbon dioxide diffusion and vapour exchange, leading to enhanced photosynthesis and promoted transpiration. Although in photoautotrophic culture conditions it has been found that increased CO_2 levels did result in enhanced growth and more efficient establishment upon transfer to the field (for review see Kaur 2015), this required active elevation of CO_2 levels either inside the culture vessels or in the growth chambers, sometimes accompanied by the complete absence of added sucrose. In our experiments, such conditions were not met and we consider the role of an increase in photosynthesis because of an increase in CO_2 availability as marginal. The net photosynthetic rate was probably not increased but still the biomass was promoted under higher air movement condition as was also found by Tay et al. (2000). We hypothesize that probably the higher level of water translocation and water transpiration, induced by enhanced air exchange through the lid filter, might be the main reason for the increased biomass accumulation in culture (Figure 1). Air

movement might decrease humidity in the vessel and improve the transpiration rate (Niu and Kozai, 1997; Aitken-Christie et al., 2013). Still we do not fully exclude the potential enhancement of growth being in part driven by promotion of photosynthesis under conditions of increased air exchange.

Transpiration and its role in *in vitro* culture

Because the gas exchange rate showed a positive relation with growth (Figure 1), we were interested in finding further evidence for the occurrence of transpiration in tissue culture under conditions of high relative humidity and in determining whether transpiration had an essential impact on element migration. Intact shoots with leaves and potentially more transpiration showed significant more translocation of acid fuchsine (Figure 2) indicative of higher water and nutrient migration internally within plants. The difference in acid fuchsine translocation between intact shoots and defoliated shoots was thought being caused by leaf transpiration (Figure 2). Herewith, we confirmed the possibility of transpiration taking place even in high humidity conditions *in vitro* and verified that *in vitro* leaf transpiration could affect the translocation of nutrients. The transpiration rate, in fact, in tissue culture is commonly believed as being marginal (de Klerk and Pramanik, 2017), a few percent of that *ex vitro* (de Klerk, 2010) and thus it is not a surprise that micropropagated plants are limited in growth by insufficient absorption of water and carbohydrates from the medium. The results here suggested the presence of transpiration *in vitro* which supported significant element translocation inside plants. However, transpiration rates might still be far lower compared to the normal *ex vitro* situation. We suppose that the low uptake of nutrients is associated with low internal water movement in plants, but that the translocation of medium nutrients could very well be promoted by enhancing transpiration.

Transpiration increase by reducing the relative humidity and its effect on growth

We reduced the relative humidity inside culture vessels by the addition of a hygroscopic solution of saturated potassium chloride. After growth under these conditions, the water retention capacity was tested and was found to be reduced (Figure 3). Plants with a higher level of transpiration also showed higher dry weight accumulation (Figure 3) and better growth. The relative humidity in *in vitro* conditions generally considered to be quite close to 100% in a conventional vessel (Kozai and Kubota, 2005) impairing transpiration and hereby inhibiting sufficient nutrient translocation. Martin et al. (2007) considered this to be the main reason for the observed inadequate absorption and translocation of cations, such as calcium, causing tip burn and shoot necrosis *in vitro.* Nutrient element migration is driven by diffusion and "hitching with any water flow" in conventional high humidity culture systems (de Klerk, 2010). However, the experimental evidence for the effect of humidity on nutriment migration is scarce. As for container humidity relative humidity in tissue culture vessels could be lower than 100% for most of the time, ranging from 90% to 99.5% (Kozai et al., 2005; Chen, 2004; Saher et al., 2005), meaning that residual transpiration could still contribute significantly to nutrient translocation, much more than generally expected. Relative humidity in vessels with an air-permeable filter is equivalent to 80% - 90%; 80% on day 2 and 90% on day 24 after plant transfer (Kozai et al., 2005). The transpiration rate at a relative humidity of 80% is 10 times higher than that of 98% at the same air temperature (Kozai et al., 2005). Our results also demonstrated that elevated air exchange rates and reduced relative humidity

can lead to better growth parameters in terms of size and weight. The relatively high but still unsaturated humidity in vessels allows transpiration both by water loss through stomata and through the cuticle of plant leaves, both of which could provide the driving force for element migration. Growth of *in vitro* plants may be improved by optimization of vessel parameters, chamber parameters and by the application of new techniques, of which the most commonly used one is relative humidity reduction in culture vessels (Wardle et al., 1983; Smith et al., 1990). To achieve this, various techniques have been used such as removing container lids for a period of time, covering vessels with more permeable covers (Cournac et al., 1991; Zobayed et al., 2000; Zobayed, 2005; Wardle et al., 1983; Short et al., 1985; Tanaka et al., 2005), using bottom cooling ventilation (Ghashghaie et al., 1992), adding salt-saturated solutions (Driver and Kuniyuki, 1984; Tanaka et al., 1992; Cha-um et al., 2003; Cha-um et al., 2010) and using glycerol solutions in the culture room (Forney and Brandl, 1992). Our research confirmed earlier results on the importance of humidity and transpiration for plants growth and provided a new option to optimize plant growth.

The effects of enhancing stomatal and cuticle transpiration on growth

We confirmed the importance of transpiration in tissue culture and we were interested whether transpiration could be enhanced, e.g. through manipulation of stomatal opening and whether this might promote plant growth. The effect of δ-aminolevulinic acid (ALA) on inducing stomatal opening was established in our experiments and water loss as a measure for transpiration was greater in shoots exposed to ALA than in untreated shoots (Figure 4 and 5). This concurs with the fact that ALA inhibits ABA-induced stomatal closure, promoting air diffusion in the stomatal cavity, thus being positively correlated with *ex vitro* plant growth and yield (Nishihara et al., 2003; Liu et al., 2011; An et al., 2016). Treatment with exogenous ALA increased both stomatal pore area and stomatal aperture width as well as seedling growth in alfalfa (Han et al., 2018). ALA has been demonstrated to facilitate stomatal conductance in melon (*Cucumis melo*) (Wang et al., 2004) and to reduce stomatal closure in date palm (*Phoenix dactylifera*) (Youssef and Awad, 2008), apple (Gao et al., 2013) and many other crops. Reports also showed that stomatal opening (aperture size) is an important factor in determining photosynthesis and plant growth (Lawson and Blatt, 2014; Wang et al., 2014) *ex vitro*. However our results demonstrated that ALA induced stomatal opening and increased shoot size *in vitro*, where shoots were heterotrophic with generally inferior photosynthesis. Promoted stomatal aperture facilitates vapour transpiration and it is likely to accelerate translocation of water and with that also translocation of dissolved nutrient, such as carbohydrates and minerals *in vitro*. Tsukamoto et al. (2004) also found that ALA enhanced $H_2{}^{15}O$ translocation in rice (*Oryza sativa* L. cv. Nipponbare) *ex vitro* as monitored by positron emitting tracer imaging. It has been demonstrated that ALA substantially promoted nutrient absorption such as phosphate (Yao et al., 2006; Ali et al., 2014) and sulphur (Ali et al., 2014) *ex vitro*. In our experiments, ALA induced apple shoot growth leading to a higher fresh weight, however, not to higher dry weight *in vitro* (Figure 5) possibly indicating that water translocation and nutrient elements translocation might be partly independent *in vitro*. Even so, some reports demonstrated that the assimilation of nitrogen (Wei et al., 2012), magnesium and copper (Naeem et al., 2010; Zhang et al., 2015) was significantly increased in ALA-treated plants *ex vitro*. Better yield and growth, production have been associated with ALA treatment (Air et al., 2018). This fact that ALA induced stomatal opening and plant growth agrees with our observations

that an increase in stomatal density in the Arabidopsis mutant *epf1epf2* facilitated growth *in vitro*.

The root system is the organ through which plants obtain nutrient elements and water (Sharp et al., 2004). ALA overexpressing transgenic *Arabidopsis* (YHem1) and plants where ALA was exogenously applied revealed a greater root dry weight and improved root length than control or untreated plants (Kosar et al., 2015; An et al., 2016). The accelerated root activity and root biomass might be attributed to growth induced by ALA (Sharp et al., 2004). In our research, micropropagated apple shoots form callus at their base and this is the place for nutrient uptake instead of roots. In addition, it is known that aquaporins are responsible for membrane water movement and hence contribute to root water uptake and leaf transpiration (Moshelion et al., 2015; Maurel et al., 2016; Gambetta et al., 2017; Maurel and Prado, 2017). ALA application induced high expression levels of aquaporins PIP1 and PIP2 in tomato seedling roots (Zhao et al., 2015). More research is required to disclose the relationship between aquaporins, callus and ALA treatment in plant species growing in *in vitro*.

For cuticle transpiration, the quantity, composition and structure may be of importance (Riederer and Schreiber, 2001). In a previous study by us, Arabidopsis *att1* mutants had a damaged cuticle, and showed increased water loss and increased growth *in vitro*. We wanted to confirm this significant contribution of cuticular transpiration to growth in apple *in vitro* shoots as well, but lacked similar mutants. Hence, we followed two other approaches. Metolachlor, categorized as an α–chloroacetamide chemical, is utilized in agriculture as a pre-emergence herbicide for effective weed control (Devlin et al., 1983; Braverman et al., 1985; Teasdale and Colacicco, 1985). Although the precise mechanism is not known, the metolachlor herbicide is thought to inhibit cuticle formation by interfering with the plastidic very-long-chain-fatty-acids (VLCFAs) biosynthesis pathway (Gronwald, 1991). These VLCFAs with a carbon chain even longer than 22 are precursors of suberin, wax and cutin, which protect plants from desiccation and external aggression (Harwood, 1996; Kihara, 2012). However metolachlor treatment of apple shoots did not show any increase in fresh or dry weight *in vitro* and no increase in transpiration. (Figure 6). Unfortunately, we were technically not able to demonstrate a reduction in cuticle formation as a result of the metolachlor application. So, we cannot rule out that the herbicide did not have the assumed effect on cuticle formation leading to the observed lack of phenotype. Nevertheless, other cuticle formation inhibitors could be beneficial to promote transpiration, such as trichloroacetate (TCA) or EPTC (S-ethyl dipropylthioarbarnate) (Dewey et al., 1956; de Klerk and Pramanik, 2017). EPTC impaired the proper cuticle arrangement on corn leaf surfaces and caused a higher cuticular transpiration *ex vitro* (Leavitt and Penner, 1979). Apart from this cuticle removal by rubbing with solvents, such as chloroform, hexane and diethyl ether (Figure 6) proved unsuccessful in increasing transpiration and shoot growth. The treated leaves became brownish and wilted in two days as shown in Figure 6. In summary the assays we tried were not sufficient for modulating cuticle transpiration in an attempt to stimulate *in vitro* growth of apple shoots.

Conclusion

Growth of apple *in vitro* shoots can be positively promoted by improving transpiration, which might be potentially modulated through a stomata opening inducer such as ALA or by increasing gas exchange rates using specific covers. Our results confirmed that relative humidity might be controlled by potassium chloride or by the filters differing in gas exchange rate, leading to improved transpiration and better growth. We demonstrated this earlier using Arabidopsis seedlings, and here again in apple shoots. Still, we need further studies to verify whether these measures apply to a broader spectrum of species *in vitro* and can be used on a larger, commercial scale.

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General discussion

Chapter 6

The growth of *in vitro* microplants as determined by the culture medium

A successful establishment or sustainment of an *in vitro* micropropagation procedure in a crop is related to medium type and composition, amongst others with respect to the necessary nutritious ingredients. Induced cell or tissue growth *in vitro* is highly dependent on the exogenous supply of nutrients, and their translocation to and within plants, and later its utilization. The *in vitro* plant growth response is known to vary, from one container to another and from one species to another, because plants in a container in a climate chamber are influenced by many external (environmental) and internal physiological factors. Factors affecting growth performance include culture medium salts, sugar composition and concentration, pH, microplant features, container type, and climate chamber conditions, all of which determine culture establishment and propagation efficiency.

The culture medium, in which plantlets are placed, plays an important role in *in vitro* plant growth, multiplication and final acclimatization to *ex vitro* conditions (George et al., 2008b). Both in liquid culture as well as on solidified medium, plant biomass accumulation and energy consumption rely on the supply from the medium and its subsequent utilization. The medium is generally composed of mineral salts, organic nutrients, vitamins and specific phytohormones profoundly influencing cell division and cell elongation. Mineral salts in the medium of which the most widely used one is Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), include macro-nutrients (nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), and sulphur (S)) and micro-nutrients (iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), boron (B), copper (Cu), molybdenum (Mo), and zinc (Zn)). The final mineral composition in the gellified substrate however might change as precipitation occurs in an endothermic process when the medium is being autoclaved (George et al., 2008a). Additional micro-nutrients might be supplemented to the medium via the agar/gelrite which can contain some impurities such as nickel (Gerendás et al., 1999). Plant morphogenesis and growth *in vitro* can potentially be regulated by inorganic salts (Ramage and Williams, 2002; George et al., 2008a). Inorganic salts may affect the rooting pattern and root architecture because of their function as signalling agents (López-Bucio et al., 2003). For such reasons, it is recommended to include the micro-minerals copper, cobalt, nickel, titanium and beryllium in culture medium (Berthelot, 1934; Nobécourt, 1937; Gautheret, 1939). Micro-nutrients iron, boron, manganese, zinc and copper were also found to be beneficial for growth in tissue culture (Heller, 1953; George et al., 2008a) and zinc, molybdenum, manganese, and copper, proved necessary for root growth and development in tissue culture (Eltinge and Reed, 1940; Glasstone, 1947; Hannay and Street, 1954).

Sugar as carbon source has an inevitably direct and obvious influence on *in vitro* growth, various metabolic processes, and propagation process (Kubeš et al., 2014). Sucrose might reduce the chlorophyll level and enzyme activity for photosynthesis thus limiting photosynthetic efficiency (Hazarika, 2006). High concentrations of sucrose available in the culture medium can lead to significant stress to plantlets as visualized by an excess production of anthocyanins (Dai et al., 2014). However, too low concentrations of sucrose will result in restricted or retarded growth as was observed in culture in complete absence of sucrose from the medium because the aerial carbon dioxide used in photosynthesis under *in vitro* conditions inadequately supports autotrophic development

(Jo et al., 2009). Apart from inorganic nutrients and sucrose, both generally supplemented in the artificial culture medium, other components are sometimes added to the medium, being rather undefined by nature, such as yeast extract, coconut milk, and banana homogenate (Arditti, 2009; Chugh et al., 2009), as a proxy of carbon and energy source, and other organic materials such as amino acids and vitamins.

Plant tissues can tolerate pH in a range from 3 to 8. If medium pH is lower than 3 or higher than 8, the inoculated tissues will mostly stop growing and eventually die (Butenko et al., 1984). However, with the medium pH at the optimal level, the availability, solubility and utilization of medium ingredients can be improved (Pasqual et al., 2002). Uptake of medium ingredients including growth regulators, inorganic salts, vitamins and hormones, can be governed by medium pH *in vitro* (Kubeš et al., 2014). Generally, when the pH is lowered (acid is in excess) negatively charged ions are easily taken up while under alkaline conditions positively charged ions are favoured. The uptake of nitrate and ammonium are influenced by acid or alkaline in the medium as well as root/callus growth (Nickell and Burkholder, 1950; Sheat et al., 1959; Asher, 1978). Intracellular biochemical reactions in plant cells and adventitious root induction on excised plant tissues were found to be dependent on an optimum value of pH (Harbage et al., 1998; Felle, 2001; Sakano, 2001; De Klerk et al., 2008; Parveen and Shahzad, 2014). A medium substrate which is too acid or alkaline influences culture initiation (Anderson, 1975; Berghoef and Bruinsma, 1979), morphogenesis (Khan et al., 1986; Mutaftschiev et al., 1987), and embryogenesis (Smith and Krikorian, 1989; Cho et al., 2003). As a rule, based on broad-scale empirical experiences, a slightly acidic medium (pH 5.6) is favoured and leads to better results in micropropagation.

In vitro growth and morphogenesis, both in multiplication as well as in adventitious rooting, are largely governed by properties of the microplant source of which the quality and numbers can depend on the cutting season and on the physiological state (Hartmann and Kester, 1975; Rodrigues Borges et al., 2011; Parveen et al., 2015). When plant tissues are taken from stock plants at different times the capability of microplantlets growth and multiplication may differ even in the same environment (Hartmann and Kester, 1975; George et al., 2008b). Microplant growth is also related to the physical condition parameters of container and climate room.

Approaches to optimize plantlet growth by manipulating external factors

To promote cell division, explant growth, tissue regeneration or shoot proliferation in plant tissue culture, most studies focus on modifications of medium composition (Gamborg, 1991) and regulation of physical environments. Countless practical trials sometimes may be required for acquiring the knowledge about the optimal growth conditions of a particular plant species or cultivar *in vitro*. Presence of sufficient nutrients in the medium does not necessarily guarantee adequate quantities of the nutrients being delivered into plants in tissue culture. Physical processes are heavily involved in uptake and translocation of the nutrients to the parts of the plants where they are assimilated. For instance, an increased level of transpiration leads to higher amounts of dry/fresh weight *in vitro* (see Chapter 2). Physical parameters in a traditional non-ventilated vessel, such as gas replacement rate and vessel moisture levels, might affect nutrient translocation in plants *in vitro* (see Chapter 5). For achieving an optimal growth of tissues and plants the medium, which is abundant in nutrients, should be in the proper

condition for facilitating nutrient uptake and the aerial environment, which has a high relative humidity (approximately 98%) and low photosynthetic photon flux density (PPFD) (40–50 μmol m^{-2} s^{-1}), should allow transpiration to a certain level but also the photosynthetic machinery needs to operate to some extent.

High ventilation in vessels is beneficial for control of the humidity at a lower level and for enhancement of transpiration, leading to an improved rate of nutrient uptake *in vitro*. It has been suggested that the relative humidity can be actively controlled and lowered from the generally expected 98% to more growth-effective 88%-94% levels and even less (Tanaka et al., 1992; Altman and Loberant, 1998). Plant growth can be promoted by increasing the gas permeability of the vessel and stimulating effective ventilation, e.g. in *Myrtus communis* L. (Lucchesini et al., 2006), in *Momordica grosvenori* (Zhang et al., 2009) and in *Gerbera jamesonii* L. (Liao et al., 2007). Apart from using gas permeable membranes in the lids of growth vessels, the internal humidity can be altered by applying higher concentrations of gelling agents and by careful management of the growth chamber humidity. The latter can also help to decrease the level of contamination and hyperhydricity when humidity of the climate chamber is brought at a level lower than 85% (Loberant and Altman, 2010).

In autotrophic plant cultures the concentration of CO_2 in the aerial environment needs to be finely regulated. The CO_2 concentration inside containers which contain plants with active chlorophyll, decreases after the onset of the photoperiod and is proportional to the light radiation (Afreen-Zobayed et al., 1999). To maintain the ability to photosynthesize and to sustain efficient growth, the CO_2 concentration should be kept above the compensation point of *in vitro* plants. To achieve this, the CO_2 concentration in the climate chamber as well as the air exchange rate in the containers must be elevated during the photoperiod (Niu and Kozai, 1997). For this, air distribution systems should be build-in in the growth chambers ensuring an even distribution of the CO_2. Gas-permeable-filters in covers have been used and this resulted in the necessary increase of gas exchange between the inside and the outside of a vessel, and a subsequent elevation of the CO_2 concentration in vessels during the photoperiod, and, as a consequence, an improved growth rate (Cui et al., 2000; Kitaya et al., 2005).

Only a limited number of plants can be cultured autotrophically (George et al., 2008c) and even can be propagated commercially in this way (Kozai, 1991). An important factor in autotrophic culture is the light for photosynthetic carbon fixation. Parameters to consider when looking at plant growth *in vitro* are light intensity and availability of wavelengths active and usable for photosynthesis (Tanaka et al., 1998; Dubuc and Desjardins, 2004). However, radiation should not become too high under conditions where the concentration of CO_2 is too low and limiting for photosynthesis because of the chance of inducing oxidative stress and *in vitro* plant sensitivity. Mostly used at present in *in vitro* culture are fluorescent TL lamps but light emitting diodes (LEDs) are more preferable because LEDs have a better radiation profile for active photosynthesis as well as a higher energy conversion capability (Tan Nhut et al., 2001; Gupta and Jatothu, 2013; Tùng et al., 2016); LEDs will become more and more used *in vitro* in the near future is my expectation.

Apart from ventilation, CO_2 concentration and light, the most important factor for growth in tissue culture is medium composition with ingredients which provide energy, sources of carbon and osmotic agents. First, for a few plant species the net photosynthetic rate is

adequate to provide photoautotrophic energy and carbon. Sugar is not necessarily added in medium here and as a result microbial contamination is reduced and less loss of production can be expected. Secondly, although sucrose is the best carbohydrate source for many plant species, other sugars like glucose induce better shoot production and multiplication rates in plant species of the genus Alnus such as *Alnus crispa*, *Alnus cordata* and *Alnus rubra* (Tremblay et al., 1984; Tremblay and Lalonde, 1984; Barghchi, 1988). Without glucose, the growth of isolated roots of wheat would be impossible (Ferguson, 1967). Shoot growth and axillary shoot regeneration have been stimulated in *Castanea* when sucrose is replaced by fructose (Chauvin and Salesses, 1987). Autoclaved sucrose medium induces better plant growth in tissue culture rather than filter sterilised sucrose (White, 1932; Ball, 1953; Johri and Guha, 1963; Guha and Johri, 1966; Verma, 1971). This supports the impression that glucose or/and fructose are beneficial for tissue growth as the autoclaving process might lead to the partial breakdown of sucrose into the monohexoses glucose and fructose. Thirdly, small amounts of defined or undefined supplements are recommended to be added to the medium to improve plant tissue growth and stimulate morphogenesis. Supplements such as coconut milk or fruit juice provide trace amounts of certain undefined organic compounds. Homogenised banana fruit is added to the medium for orchid regeneration and growth promotion (Chugh et al., 2009). A specific special additive is *myo*-inositol which is required for *Fraxinus pennsylvanica* callus to achieve maximum growth (Wolter and Skoog, 1966). Riboflavin is used to promote adventitious rooting on shoots in *Carica papaya* (Drew et al., 1993), apple (van der Krieken et al., 1992) and *Eucalyptus globulus* (Trindade and Pais, 1997). Finally, the purpose of culture should be considered when choosing liquid medium or gelled medium. When it is for the culture of protoplasts, excised root systems, or for the production of secondary metabolites in cell systems, obviously liquid medium (e.g. in a bioreactor) is used which allows a faster growth rate because cultures are immersed directly and fully in nutrients. However, full and continuous submersion in liquid can only be applied to whole plants when they are un-sensitive to hypoxia and hyperhydration. To avoid this, great progress has been made by using so-called temporary immersion bioreactors. Plants are then submersed only for a fixed and limited time period, allowing efficient uptake of nutrients by the entire plant and all its organs. Otherwise solidified media with supporting matrices are to be preferred with a low chance of hypoxia or hyperhydricity.

Approaches to enhance *in vitro* plantlet growth, the plant's perspective

As mentioned in the previous section, conventional, relatively airtight vessels, in which cultures are established, have a high relative humidity, or, in other words, a low vapour pressure deficit and a strongly-reduced water diffusion rate. Transpiration is restricted by this higher relative humidity (Ehret and Ho, 1986; Gisleröd et al., 1987) and the low air exchange caused by sealing of the vessels greatly reduces water translocation in plants and this likely results in limited internal nutrient transport (White, 2012). Water translocation in xylem is closely related to or even almost exclusively driven by transpiration; a controlled moderate humidity ensures sufficient transpiration; reducing humidity enhances axial transport of inorganic nutrients in plants (Torre et al., 2001). Migration of water as well as inorganic nutrients from roots to shoots *ex vitro* is attributed to plant transpiration (Bouranis et al., 2014). I propose that the "general impression" that transpiration *in vitro* is negligible and inadequate to generate any force

driving nutrient translocation from medium to the shoots is wrong. Below I will outline why this in my view is the case.

In **Chapter 5** we investigated whether transpiration as such occurred in tissue culture. We found that the vials containing shoots showed a reduction in weight in a few days demonstrating the water loss through shoot leaves *in vitro*. Water loss by transpiration took place in plant tissue culture even under high humidity conditions (90%-95%). At the same time, we were interested in determining whether water loss was closely related to element flow in tissue culture. The point was to find out whether elevated transpiration might give rise to a boost in nutrient transport within *in vitro* plants. Apple microplants without leaves showed a slow and restricted flow of an acid fuchsine solution*,* whereas intact explants with leaves demonstrated a quick distribution of acid fuchsine over a longer distance within the plantlets. Acid fuchsine was utilized to mimic small-molecular-weight nutrients, such as minerals, sucrose, amino acids and plant growth regulators. With these experiments it was demonstrated that leaf transpiration is linked to uptake and also translocation of exogenously supplied sucrose *in vitro*.

The vapour pressure deficit between the surrounding air and the stomatal cavities or cuticle layer drives water molecule evaporation even in high humidity culture. We suppose that most transpiration occurs through stomatal cavities while only a part of the total water loss takes place via the cuticular surface of plants *in vitro*. The transpiration rate is mainly influenced by water vapour concentration gradients between the stomata and the surrounding air, and by the resistance imposed by stomata or by boundary cuticular layers (Meidner, 1975; Farquhar and Raschke, 1978; Buckley et al., 2017). The vapour concentration gradients might be low *in vitro*, but they are seldom zero. Cuticular water loss and stomatal transpiration, being the driving force of exogenous nutrients flow, are associated with the upward flow of carbohydrates in plant tissue culture, governing plant growth.

Although the improvement of transpiration and growth in cuticular and stomatal mutants has no practical importance in Arabidopsis, still using them provided knowledge on the important roles the cuticle and stomata play in plant biomass accumulation in plant tissue culture. In **Chapter 2** we investigated the effects on growth of Arabidopsis plants *in vitro* by manipulation of cuticle transpiration and stomata transpiration separately. Mutant *cer5*, which was characterized by a reduction of cuticular wax deposition by epidermal cells (Pighin et al., 2004), did not show the expected increase in in fresh weight. Still, the mutant line *cer5* did have increased cuticle permeability as shown in a chlorophyll release assay, and increased transpiration as shown in a water loss assay. This result did not provide us yet with conclusive evidence of a complete link between transpiration and growth, i.e. transpiration influenced by cuticular wax. Mutant *att1*, which was characterized by reduced cutin biosynthesis and loose cuticle ultrastructure (Xiao et al., 2004; Duan and Schuler, 2005), did show enhanced biomass accumulation. The increased transpiration in *att1* leading to elevated fresh weight and dry weight might be due to the defective cutin pathway. This provided at least some evidence that transpiration through the cuticle does play a role in determining growth *in vitro.* With respect to the contribution of stomatal transpiration, we demonstrated that the Arabidopsis mutant line *spch,* which has a severely lowered number of stomata, had significantly lower levels of transpiration as found in the water loss assay and showed less fresh weight accumulation while the mutant line *epf1epf2,* which has an almost doubled number of stomata compared to wildtype, showed increased water loss and

biomass accumulation. So in our study, the positive effects of cuticular transpiration and stomatal transpiration, on plant biomass accumulation, growth, *in vitro* were established.

We hypothesize that in tissue culture transpiration, water loss via plant leaves, is a prerequisite for nutrient solute transport, whether inorganic or organic, through which they can arrive in the uppermost parts of plants via the vascular system. As source of carbon, sucrose can be considered as the main determinant of growth and propagation *in vitro*. Although abundant levels of nutrients are available in the medium, the absorption of elements in roots as well as the upward translocation is dependent on water moving upwards. Optimization of growth and multiplication rates by commercial *in vitro* propagators could be achieved by measures to increase transpiration.

Translocation of exogenously supplied sucrose *in vitro*

Generally in plants grown under *ex vitro* (or *in vivo*) conditions, sucrose translocation is driven by osmosis and the water potential gradient existing between source and sink tissues. Here, sucrose is loaded into sieve elements in leaves (source), causing turgor pressure in the sieve tubes as the force driving the flow, and the unloading in sink tissue (roots, flowers and seeds), by allowing the efflux of water. Sucrose translocation over long distances along the plant axis is caused by phloem hydrostatic pressure. For short distance translocation, primarily occurring radially, sucrose, once formed by photosynthesis in mesophyll cells, is transported to phloem parenchyma cells, and subsequently into the sieve element-companion cell complex (Oparka and Turgeon, 1999; Liesche and Patrick, 2017). Sucrose efflux from mesophyll cell to into the apoplast is regulated by SWEET genes but its uploading in sieve element-companion cells is regulated by SUC genes (Sauer, 2007; Chen et al., 2012). Sieve elements are connected end to end and form sieve tubes where sucrose is translocated over long distances. Arriving through the phloem at the sink, the sucrose is unloaded either through plasmodesmata along cytoplastic continuums, the predominant route, or by an apoplastic route along cell wall structures (Lalonde et al., 2003; Schulz, 2005; Zhang et al., 2006), or by a transcellular path across cell membranes. In the latter two paths, sucrose translocators play again a role.

To support and maintain plant growth the tissue culture medium can be supplemented with various sugars, but mostly with sucrose as the main form of carbohydrates. However up till now, only a limited number of studies have been devoted to the mechanism of how medium sucrose is translocated or which candidate transporters regulate translocation from the medium into and through plants. In **Chapter 3** I investigated the physiological role of SWEET11 and SWEET12 in uptake and transport of exogenous sucrose in tissue culture. Single knock-out mutants showed that SWEET11 and SWEET12 might compensate each other's function, however, the double mutant *sweet11;12* showed restricted sucrose translocation. That was why *sweet11;12* demonstrated reduced fresh and dry biomass accumulation growing on normal medium while single mutants did not. I tested whether the sucrose in the medium had some influence on root length: growth of the main root in length was inhibited by increased sucrose supplementation whereas the primary roots of *sweet11;12* were less affected in growth under these conditions. It was hypothesized that the ample availability of sucrose led to a reduction in primary root length *in vitro*. The sucrose content in roots of plants was examined showing that *sweet11;12* maintained higher concentrations of sucrose than Col-0 when sucrose is applied in the medium. Probably, upward sucrose

translocation has been impaired in the double mutant *sweet11;12.* A GUS localization assay indicated that the SWEET11 and SWEET12 genes were induced by sucrose availability in roots in medium and that both genes were expressed in the vasculature. An analysis using isolated root cultures suggested that the mutations in *sweet11;12* might lead to a reduction of sucrose uptake in roots, resulting in reduced biomass accumulation. The roles of SWEET11 and SWEET12 were studied at the cellular level with respect to the sucrose translocation process and for this we attempted to demonstrate decreased uptake of esculin, the fluorescent sucrose analogue, in protoplasts of *sweet11;12* but failed. From our experiments, I deduce that SWEET11 and SWEET12 are involved in mediating exogenous sucrose uptake and translocation in roots from surrounding medium and in the later upward export process.

Anatomically, *in vitro* roots when checked along the radial direction have the same structural organization as *ex vitro* roots: epidermis, exodermis, several layers of cortex parenchyma cells, and then the endodermis and vascular cylinder. I suppose that the sucrose concentration in the apoplast is higher in the outer layer than in the inner layer in roots forming a sucrose gradient along cell wall structures. A thermodynamic force, caused by the sugar concentration gradient or by water pressure, pushes sucrose towards the endodermis. The specific composition of the apoplast of exodermal cells, with hydrophobic lignin, lipophilic suberin and the Casparian strip being there, prevents the further passive flow of the dissolved sucrose. At this point, the sucrose might have to leave the apoplast and enter the cell-to-cell route and penetrate across endodermal membranes where SWEET11 and SWEET12 are located to regulate transmembrane translocation in roots *in vitro*. Arriving at inner endodermal layer in roots sucrose might be released again from the parenchyma cells into the apoplastic area, which process is also likely regulated by particular SWEET proteins; for the final re-entry in companion cell-sieve elements SUC proteins are responsible. Then, sucrose arrives at the vascular system. Once in the sieve tube system sucrose translocation is driven by hydrostatic mass flow forces and thus reaches the upper parts of the plants at the sink site.

In the previous section I discussed sucrose translocation by the apoplast and by transmembrane routes in *in vitro* roots. The third, symplastic route might also play a role in conditions or places where sucrose flows down a concentration gradient via plasmodesmata from root hairs through epidermal cells to the vascular cylinder in roots. This passive migration does not require energy expenditure and but has a low diffusion rate. The latter, together with the paucity of plasmodesmata will greatly affect the efficiency of symplastic pathway transport and renders it unlikely that this route plays a major role in sucrose translocation.

Now how to explain the supposed inversion of the flow direction in the phloem in *in vitro* plantlets, here from bottom to top instead of from top to bottom. Sucrose solubility and its diffusion rate in gellified medium reduce the water potential, increase the osmotic pressure and form concentration gradients, particularly in areas directly neighbouring the roots. Sucrose diffusion in gellified medium, where the concentration of gelling agents and porosity play a role, is subject to thermodynamic kinetics. It is thought that sucrose diffuses passively from adjacent areas in the medium to the epidermal layer of roots. Subsequently, sucrose goes through epidermis, exodermis, cortex parenchyma cells, arriving at the endodermis and vascular cylinder. As mentioned, sucrose translocation is facilitated by sucrose transporter proteins and moves into the phloem vessels in roots. This causes a decrease of the sucrose potential along the phloem in roots *in vitro* and to

compensate for this water migrates from xylem tubes to phloem tubes due to the induced water concentration gradient. As increased amounts of solutes arrive in the lower part of the phloem tube, hydrostatic pressure could be enhanced in the phloem of roots. This pressure causes the onset of solution flow; sap moves upwards inside the shoots towards the aerial parts and finally incoming exogenous sugars are unloaded in shoots either through plasmodesmata or through the apoplastic route or via the transcellular membrane route. The unloading of sucrose produces a high water potential in the phloem in the sink areas, and thus water diffuses out, eventually going back to the xylem by osmosis for a new circulation. This process is illustrated in Figure 1.

The entire process of sucrose translocation from areas in the medium adjacent to the epidermal layer in roots to the phloem vessels is a complex one involving many steps and components (see Figure 1). It is, amongst others, assumed that sucrose transporters that are located on the membranes of cells in the endodermal layer have two functions, one to translocate sucrose from the apoplast into the cells and one to transfer sucrose back to the apoplast after having passed the block presented by the Casparian band. The sucrose also might follow the cell-to-cell pathway in the inner cell layers. The sucrose has to pass inner cell layers such as the pericycle and phloem parenchyma, before finally ending up in the phloem tube. The sucrose transporters involved may differ based on their location and function (uploading or unloading).

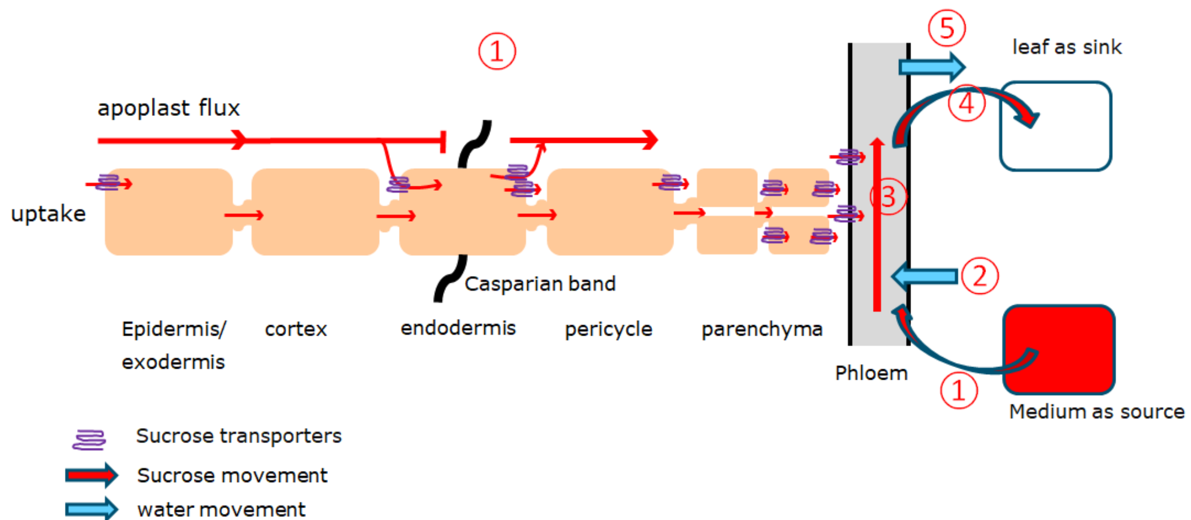

Figure 1. The process of uptake of exogenous sucrose from the medium and its upward translocation, as hypothesized. 1. Sucrose diffuses passively from adjacent areas in the medium to the epidermal layer of roots and finally moves into the phloem vessels with the help of sucrose transporters. Once inside roots, sucrose passes through epidermis, exodermis, cortex parenchyma cells, arriving at the endodermis and vascular cylinder. SWEET genes are thought to be primarily located in endodermal cells and parenchyma cells. The Casparian band blocks the flow of sucrose via the apoplastic pathway in the endodermal cell layer, however not completely. 2. Because of the sucrose entering the phloem, the water potential decreases there. As a consequence, water is recruited into the phloem from neighboring cells or xylem tissue due to the water concentration gradient. 3. Excess sugar in the phloem tube leads to hydrostatic pressure in the phloem. Pressure at bottom drives a sap stream upwards to the top of the plant where the hydrostatic pressure is low. 4. Translocated sugars are transported out of the phloem by

unloading. The unloading of sucrose likely requires sucrose transporters. 5. The active unloading of sugars results in a higher water potential in the phloem tube compared to the surrounding tissue, leading to water passing out, returning eventually to the xylem or other cells.

Translocation of water *in vitro*

Transpiration, which generates the negative pressure for transport of the bulk of the water absorbed from the medium, can occur thanks to a continuous water stream along potential gradients via the vascular system, originating from roots after water uptake, flowing through the xylem, to the stomatal cavities for vapour diffusion. The dead and hollow xylem vessel elements form a passage by which water and dissolved minerals are delivered to the shoots over a long distance. This continuous water stream throughout the plant is the result of transpirational water tension, cohesion of water molecules by hydrogen bonds, water-cell wall adhesion and the water potential gradient (Dixon, 1914; Tyree, 1997; Steudle, 2001). The radial water transport happens in concentric root layers from epidermis, going through exodermis, cortex parenchyma cells, and endodermis to the vascular cylinder. *Ex vitro* plants get their carbon and energy from leaves while obtaining water and inorganic salts from the roots. In *in vitro* plants both the uptake of elements such as water and inorganic nutrients, as well as carbon and energy takes place by the roots. In *in vitro* conditions*,* the water radial movement in roots is of importance for the upward translocation of sucrose and other salts, acquired from the medium, to the shoots. The sucrose/solute translocation in roots *in vitro* is supposed to be closely linked to the translocation of water. For the transmembrane pathway, the water transporters play an active role. I wanted to study how water translocation across semipermeable cell membranes occurs *in vitro* where aquaporin PIPs mediate this process in roots along existing water potential gradients. Water potential gradients might be formed in tissue culture containers by low vapour pressure deficit, the water status of plants, and water content of the gelling medium (Sallanon and Coudret, 1990).

Although the notion that aquaporins function as water channels is well accepted, the role of aquaporins in facilitating water flux *in vitro* and mediating plantlet growth in tissue culture is still unclear as well as its link to sucrose flux. In **Chapter 4** experiments were carried out to identify physiological functions of plasmamembrane PIP1s *in vitro.* The studies on determining its role in regulating water permeability and plant growth, were done using Arabidopsis lines in which PIP1 genes were down regulated by microRNAs. We found that increasing sucrose present in the medium induced down regulation of aquaporin PIP1 gene family members. This might explain that exogenous carbohydrate application was negatively involved in determining water permeability and transpiration. Focussing first on leaves, the hydraulic conductivity was monitored and compared with PIP1 gene expression (Cochard et al., 2007). The transpiration assay did not show any significant difference between wild type and aquaporin PIP1s silenced plants *in vitro*. This result is different from previous reports, in which expression of PIP1;2 by antisense constructs was lowered, showed a lower membrane hydraulic conductivity in isolated protoplasts and reduced root hydraulic conductivity (Kaldenhoff et al., 1998; Martre et al., 2002), and suppression of PIP1s expression in tobacco caused lower water permeability of protoplasts, and a lower transpiration level *ex vitro* (Siefritz et al., 2002; Siefritz et al., 2004). That we could not demonstrate a difference in transpiration might be related to the *in vitro* conditions where plants lose the control over their water retention capability to some extent. Shifting to the roots, water permeability at the cellular level might be

affected by sucrose: sucrose has been shown to modulate hydraulic conductivity in roots in a light-dependent manner (Di Pietro et al., 2013), and the activity of aquaporins is affected by sucrose through phosphorylation on membranes (Niittylä et al., 2007). Generally, autoclaved sucrose is provided by *in vitro* media, where roots take up the nutrients. I found that the sucrose content in roots of PIP1s silenced lines was higher than that of wild type plants implying that the activity of PIP1s might indirectly affect sucrose upward export from the roots *in vitro*. Plants with silenced PIP1 genes also showed impaired growth and reduced biomass accumulation *in vitro*. Inhibition of sucrose translocation is probably the reason for the observed inferior growth in PIP1s silenced lines. The translocation of exogenous sucrose in roots *in vitro* is assumed to take place primarily via the apoplastic and cell-to-cell routes. Water transport in roots in hydroponic culture (sugar absent) by the cell-to-cell pathway was confirmed to occur in an aquaporin-dependent manner (Knipfer et al., 2011; Sutka et al., 2011). The aquaporin-related cell-to-cell pathway dominates the water transport in roots by 53%; approximately 26% of the water transport takes place via the apoplast (Knipfer et al., 2011; Ranathunge and Schreiber, 2011; Ranathunge et al., 2017). The active cell-to-cell pathway is apparently more dominant in water flow in roots in the presence of an osmotic gradient, rather than the passive route through the porous apoplast (Steudle and Peterson, 1998; Steudle, 2000). It is unclear whether PIP1s also dominate root water flow in propagation conditions *in vitro*, and whether exogenous sucrose translocation in roots *in vitro* occurs more via the cell-to-cell route than via the apoplastic route or perhaps through combination of the two. Cultures of excised roots in containers showed a reduction in root growth in the PIPs silenced line compared to the wild type. The growth of isolated roots is dependent on uptake of nutrients from the medium and their internal translocation. The inhibited sucrose uptake and assimilation in PIP1s silenced lines is probably the reason for the growth reduction of excised roots. In summary, I hypothesize that PIP1 genes located in plasma membranes in roots might regulate sucrose translocation and plant growth through influencing across-membrane water flux.

Our interest was to reveal the interaction between root hydraulic water conductivity via membrane aquaporins and nutrient uptake *in vitro*. The speed of translocation of dissolved nutrients in plants in containers is affected by the axial water flow and the radial water flow *in vitro*. Axial flow occurs in xylem where the longitudinal resistance is close to zero in the dead ducts (Barrowclough et al., 2000). The major limiting step of hydraulic conductance occurs in the radial flow in roots *in vitro*. Although we demonstrated the effects of water transport mediated by aquaporins on growth *in vitro,* their role in the uptake and distribution of necessary nutrients along the radial axis in roots remains to be determined further. The facts that higher expression of aquaporins induced by silicon and ammonium *ex vitro* increases root hydraulic conductance and induces optimized growth performance in response to osmotic pressure (Liu et al., 2014; Ding et al., 2015), might provide a clue to tissue culture growers that the same might be possible *in vitro,* better growth and performance by stimulating aquaporin activity.

Approaches to improve *in vitro* growth by manipulating stomata and/or the cuticle

In **Chapter 5** we attempted various options to enhance leaf transpiration and plant growth. At the beginning we investigated the influence of the filter permeability in lids of tissue culture containers on microplant growth. An increase in gas replacement rate has effects in raising carbon dioxide concentrations to a higher level and lowering the vapour

pressure deficit. Although also *in vitro* photosynthesized carbohydrates as building block or energy source could contribute to growth to some extent, exogenously supplied sucrose is the predominant factor for energy supply and as carbon source. We demonstrated that the plant biomass accumulation, tested in four vessels by determining fresh and dry weights, was in concurrence with the level of gas exchange. This was probably due to the level of shoot transpiration. Flow of an acid fuchsine solution in apple shoots was linked to the presence of transpiring leaves *in vitro* as shown by the distribution of acid fuchsine in stem sections and leaves. We also attempted to alter shoot growth by influencing transpiration by addition of a hygroscopic potassium chloride solution inside the culture container. Indeed, the potassium chloride treatment promoted leaf transpiration and enhanced dry weight accumulation *in vitro*. Control of the humidity inside the container seems a workable option to regulate shoot growth *in vitro*. In addition to changing physical conditions inside the container, we were interested in monitoring the effect of altering stomatal transpiration or cuticular transpiration on *in vitro* shoot growth and development. δ-Aminolevulinic acid (ALA) is a potential stomata opening inducer which increases stomatal aperture size (Zhao et al., 2015; Anwar et al., 2018; Han et al., 2018) and improves chlorophyll biosynthesis *ex vitro* (Niu and Ma, 2018). It was determined if exogenous ALA also induced stomatal opening or increased stomatal aperture size *in vitro*. We confirmed that it did and found that it stimulated water loss, and promoted higher amounts of fresh weight *in vitro*. Metolachlor has been used to affect the composition or amount of wax and cutin on the leaf surface *ex vitro* (Mellis et al., 1982; Ebert and Ramsteiner, 1984; Tevini and Steinmüller, 1987). We utilized metolachlor in medium to inhibit cuticle formation, and alter water loss and growth *in vitro* but without any success. Apart from metolachlor, we investigated if cuticle removal from the adaxial side by using different solvents could lead to an increase in water loss and growth but also these experiments failed. In summary, inducing stomata opening by ALA rather than reducing the cuticle by applying inhibitors seems applicable to promote both plantlet transpiration as well as growth *in vitro*. Still, that cuticle transpiration might play a role nonetheless, was found by using Arabidopsis mutant lines affected in cuticle formation or deposition. Arabidopsis mutant *att1* (impaired in cutin biosynthesis and characterized by a loose cuticle ultrastructure) showed increased transpiration and growth *in vitro*.

The ability of stomata to close has been widely believed to be lacking in *in vitro* plants, explaining why desiccation and low survival rates take place when plants are transferred to *ex vitro* conditions without special measures (Brainerd and Fuchigami, 1982; Ziv et al., 1987; Blanke and Belcher, 1989; Shackel et al., 1990). Improper cellulose micro fibrils in epidermal cells and abnormal sodium/potassium accumulation in guard cells were suggested as causes for the lack of stomatal closure of cultured shoots (Wardle et al., 1981; Wardle and Short, 1983). However, data from our lab indicated that *in vitro* cultured shoots might have functional stomata after all, because they closed in the dark (Kemat et al. unpublished). In addition, on top of the results with Arabidopsis and ALA, we also showed a role for *in vitro* stomatal water loss in contributing to apple (*Malus domestica*) cv. 'Gala' normal growth. In the apple rootstock MM 106 50% of stomata proved capable to close at the second day after transfer to *ex vitro* although the stomata reacted late at the beginning (Vegvari, 2003). Stomata of apple shoots retain the ability to close in 90% relative humidity (Shackel et al., 1990). Stomata of *in vitro* grapes are in function and able to close to certain extent (Düring and Harst, 1996; Fila et al., 1998). Up to 80% stomata of cultured *Prunus cerasus* closed when being placed in 45% relative humidity (Marino, 1986). It seems stomata of cultured shoots are capable to open and

close *in vitro,* in short can be functional. However, the contribution of cuticular transpiration remains controversial. The individual contributions of stomata and the ratio between stomatal transpiration versus cuticular transpiration in plant tissue culture are not well understood yet. Further experiments are necessary to reliably establish the exact role of *in vitro* cuticular transpiration in determining growth.

Transpiration is determined by vapour pressure gradients between the air in the containers and the leaves of plantlets, and by the conductance of water moving from internal parts of the plants to the outside environment (Farquhar and Sharkey, 1982). Any treatment which might increase stomatal conductance or cuticular permeability or increase the vapour pressure gradient could promote leaf transpiration. The primary route of water evaporation is via stomata, therefore, for improving growth *in vitro*, main options are to increase stomatal number or optimize stomatal opening. I will at this point provide some ideas on how to achieve this in *in vitro* culture. Stomatal activity follows a diurnal pattern related to the light and dark cycle (Shimazaki et al., 2007). Total diurnal stomatal water loss could be influenced via the setup of artificial lighting *in vitro*. In some cases, the stomatal number is closely related with the transpiration level (Monson and Grant, 1989; Lake and Woodward, 2008). In Arabidopsis, the stomatal density was found to be correlated with expression of a gene called Stomagen and application of stomagen protein to seedlings increased stomatal density (Kondo et al., 2009; Sugano et al., 2010). Fusicoccin (FC), a wilt-inducing phytotoxin, is used to irreversibly open stomata and induce uncontrolled high levels of transpiration (Turner and Graniti, 1969; Turner and Graniti, 1976; Marre, 1979). Syringolin A, a virulence factor from *Pseudomonas syringae* (Groll et al., 2008), could suppress stomatal closure and promote stomatal opening (Schellenberg et al., 2010). Closed stomata can be induced to reopen by coronatin (Melotto et al., 2006; Melotto et al., 2008) even in dark conditions inducing closure (Mino et al., 1987). Whether or not all or some of the above mentioned options are suitable for application in *in vitro* culture need to be individually determined, always aiming to reach higher level of plant growth *in vitro*.

Concluding remarks and future perspective

In this study we investigated the growth of shoots under high humidity conditions in closed culture containers *in vitro.* Growth of plant tissue material is generally achieved by assimilation and utilization of carbohydrates and other nutrients from the artificial medium. Nutrient translocation in plantlets *in vitro* was found driven by water potential gradients caused by water loss, in particular stomatal and cuticular transpiration. Arabidopsis stomata and cuticle mutants which had altered transpiration levels showed enhancement of growth in the low vapour pressure deficit condition *in vitro*. The transpiration-growth theory was also validated in apple shoots: water loss accorded with biomass accumulation. Stomatal opening inducer δ-aminolevulinic acid (ALA) and the cuticle inhibitor metolachlor were used to regulate shoot transpiration and thus to influence biomass accumulation for *Malus domestica* 'Gala'. While stomatal manipulation was a success, the cuticle inhibitor had no significant positive influence on growth. Apart from growth enhancement we focused on the sucrose and water uptake process in *in vitro* roots. SWEET11 and SWEET12 are involved in the uptake of exogenous sucrose from the medium into the roots, and modulate sucrose upward transport in roots of Arabidopsis. The primary root length, root internal sucrose concentration, and esculin levels in protoplasts were examined to establish the influence of SWEET11 and SWEET12. A double mutation in both led to impaired growth patterns in excised root culture and on

regular solidified medium. We attempted to identify the functionality of plasmamembrane aquaporin PIP1s in roots *in vitro* by measuring water permeability and the growth pattern in an Arabidopsis microRNA-silenced line in which the PIP1 genes were down-regulated. Although we did not find a significant difference in transpiration between wild type plants and PIP1s silenced plants *in vitro,* the sucrose translocation in roots and the growth were affected by silencing PIP1s. The process by which aquaporin PIP1s determine sucrose nutrient translocation *in vitro* might be through the process of across-membrane water flux.

Cell division and plant growth are important determinants of tissue culture regeneration competence which is widely exploited in the micropropagation industry (Sussex, 2008). Even after this study, there are still many abstruse details open for further investigations, such as the cellular and molecular mechanisms underlying callus nutrient assimilation from artificial medium. In our study the emphasis has been on rooted seedlings of *Arabidopsis thaliana* as research objects with some research on non-rooted, wound-callus developing apple microshoots. In such systems, the nutrient solution is assumed to arrive at the interface of the callus and medium by hitching with water diffusion. The interesting question is how *in vitro* wound callus regulates nutrient translocation, sucrose for instance, from the interface to inside the callus. Another point is whether *in vitro* callus can develop organized structures to facilitate or modulate the uptake of water and nutrients, resembling the root structure: epidermis, exodermis, cortex parenchyma cells, endodermis and vascular cylinder. We found preliminary evidence for the development of vascular tissues in the apple callus as well as of a suberin layer (data not shown). The third point is whether *in vitro* callus regeneration is dependent on the same regulators as those involved in root development. Arabidopsis mutants affected in root development might have problems in callus formation. A mutation in *Alf4* blocks initial cell division of pericycle cells (Celenza et al., 1995), and it can be assumed that *alf4* is inhibited in callus formation. To determine if callus grown in high cytokinin medium might resemble the root meristem, specific reporter genes, *p*WOX5-GFP and *p*PLETHORA1-GFP (Blilou et al., 2005), can be examined *in vitro*. Callus regeneration and root development might be compared using the root meristem marker *p*SCR-GFP and the root pericycle marker J0121 (Sugimoto et al., 2010). HB8, a marker gene for vessel formation (Baima et al., 1995; Baima et al., 2001) might be used to analyse cambium formation and vasculature regeneration *in vitro* (Mazur et al., 2016). Finally with respect to our research, it would be exciting to know whether and what aquaporins and sucrose transporters are involved in callus growth and development *in vitro*.

Culture establishment starts from freshly excised plant parts incubated in solidified medium containing abundant nutrients in a culture container. Nutritional solutes will enter the explants, such as apical shoots and stem fragments, via the exposed cut surface and all other surfaces in direct contact. The main question is how sucrose is taken up and transported via cut section, ultimately arriving at the apical top. *Ex vitro,* the plant xylem conducts water and dissolved salts up, whereas the phloem conducts organics down in the vascular system. It is unclear whether nutrient elements are transported upwards by xylem dead ducts or by the phloem system *in vitro*. After successful initial setup, the next step in micropropagation is the stage of propagule production. Fresh cut surfaces incubated in propagation medium probably generate wound tissue containing hydrophobic compounds, such as suberin and cuticular waxes. The lipophilic substances in the tissue being impermeable for water dissolved compounds, might inhibit the efficiency of nutrient uptake into microplants. Compared to uptake via

the cut surface, wound periderm formation including a ligno-suberized layer (Oven et al., 1999), might block and actively select nutrients for uptake *in vitro*. Wound stress being a required stimulus in initiating regeneration (Iwase et al., 2015), induces various cellular responses, plant hormone production (Ahkami et al., 2009), and cell-to-cell communication as well as long distance interaction (Melnyk et al., 2015). Phenolic constituents and suberin deposition on the surface might strengthen the barrier against water and sucrose uptake *in vitro*. It would be interesting to understand the potential interaction in nutrient translocation between the apoplastic route and the cell-to-cell pathway in callus *in vitro*.

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Summary

Micropropagated plantlets are grown *in vitro* on medium which abundantly contains the necessary nutrients. However, growth is far from satisfactory in conventional non-ventilated closed vessels with particular physical conditions within, e.g. high humidity, varying and insufficient carbon dioxide levels and low light intensity. The success of plant tissue culture depends on the assimilation and utilization of the available nutrients. Our study is to understand plantlet growth in tissue culture and attempt to enhance biomass accumulation in plantlets. We assessed the influence of transpiration on growth under the generally low-vapour pressure deficit conditions present in *in vitro* conditions. It was found that plantlet growth *in vitro* is closely correlated with water and sucrose uptake from the artificial medium. Therefore, it is vital to get a basic understanding regarding the determining factors of water flux and sucrose transport in *in vitro* roots as they are taken up from the medium.

In **Chapter 2** we explored the effect of transpiration on plant growth *in vitro*. The transpiration was altered in Arabidopsis cuticular mutants *att1* and *cer5*, and in stomata density mutants *spch* and *epf1epf2* and the corresponding influence on biomass accumulation was monitored. Our aim is to provide options for enhancing plant growth performance and multiplication rate. Leaves of mutant line *att1* showed an increased dye (toluidine blue) penetration, increased chlorophyll leakage, and enhanced water loss without showing any alteration in stomatal characteristics, such as density and size. We also observed an increase in fresh weight and dry weigh and larger sized plants in mutant *att1*. Mutant *cer5* had increased chlorophyll leakage and water loss whereas the biomass was not increased *in vitro*. So, the impaired wax export in *cer5* did not have a beneficial influence on biomass accumulation. We concluded that the increase in plant growth *in vitro* was correlated to a rise in cuticular transpiration but not all observations could be easily explained. Mutant line *spch,* having fewer stomata, grew stunted and weak, however mutant line *epf1epf2* having two times the number of stomata compared to the wild-type grew bigger and stronger. The stomatal density increase contributed to increased water loss and biomass accumulation *in vitro*. In summary, by looking at transpiration at the plant level and investigating the roles of the surface cuticle and of stomatal activity we suggest that growth, being the result of carbohydrate partitioning, is dependent on water flux driven by transpiration, also under high relative humidity *in vitro,* as shown in the stomata mutants and cuticle mutants. This relationship between water translocation and plant biomass *in vitro* is relevant for plant tissue culture. Transpiration *in vitro* is low but still important for growth and can be targeted for improvement measures to ameliorate water status and with this the growth in plant tissue culture.

In **Chapter 3** we investigated the mechanism of sucrose translocation focussing on the process in roots *in vitro*. Exogenous sucrose determines the plantlet's growth after uptake by the roots and subsequent translocation upwards in the plant. We collected leaves and roots *ex vitro* and *in vitro* and looked at gene expression of sugar transporters. SWEET11 and SWEET12 were selected as the candidate genes which might play a role in sucrose uptake and translocation in roots. Single *sweet11* and *sweet12* mutants did not show a noticeable change in phenotype, however the double mutant *sweet11;12* was clearly reduced in growth *in vitro*. It was observed that sucrose had an inhibitory effect on primary root length in the wild-type Col-0 and that the primary root length of *sweet11;12* was less affected by increasing sucrose concentrations. We also determined sucrose levels in roots biochemically and found that *sweet11;12* retained higher concentrations of sucrose there. These results suggested that SWEET11 and SWEET12

are jointly involved in sucrose translocation in roots and can compensate each other. A GUS staining assay showed that the SWEET11 protein and SWEET12 protein were induced by sucrose present in the medium and both proteins were located in or near the vascular system of roots *in vitro*. To rule out the influence of endogenously synthesized sugar on root growth and development as well as the influence of sugar transporters in leaves, an isolated root culture was established and used to examine root sucrose assimilation. The root culture of *sweet11;12* showed a reduction in sugar uptake, which can explain the reduction in biomass accumulation and the slight pH alteration. The role of SWEET11 and SWEET12 in sucrose transmembrane translocation was further investigated in protoplasts of mesophyll cells and root cells by studying esculin uptake, however, we did not find the expected decrease in uptake of esculin in *sweet11;12* protoplasts. We concluded that it is very likely that SWEET11 and SWEET12 participate in sucrose uptake from the medium and in sucrose migration apically in tissue culture.

In **Chapter 4** we determined the relationship between plasma membrane water flux regulated by aquaporins and plant growth *in vitro*. We employed artificial microRNA mediated gene silencing which generated small RNA sequences specifically down regulating PIP1 genes in Arabidopsis. The gene expression levels of the PIP1 subfamily in particular were examined *in vitro* and the effect of varying concentrations of sucrose on them. It was found that sucrose had an inhibitory effect on expression of PIP1 aquaporins. Visual inspection of the silenced plants learned that they were smaller in size and their fresh and dry weights were reduced in tissue culture. The sugars isolated from the roots showed that in the silenced plants sucrose was higher suggesting that the water cell-to-cell flux mediated by aquaporin PIP1s might be involved in sucrose upward translocation. The accumulated sucrose might also be an indication for the lack of a sufficient carbohydrate metabolism explaining inferior root development *in vitro*. In order to better understand the assimilation process in roots *in vitro* we again used an excised root culture and confirmed that the growth of isolated roots of silenced plants was impaired. Next, we investigated whether the reduced water transmembrane translocation in roots of the PIP1 silenced plants affected transpiration in higher parts of the plants, i.e. the leaves. However, no differences were found between the wild type Col-0 and PIP1s silenced plants *in vitro*. We demonstrated the contribution of aquaporin PIP1 proteins to plant *in vitro* growth through investigating physiological characteristics of miRNA-induced-PIP1s-silenced plants. The results showed that both sucrose uptake in roots from the medium as well as plant growth are correlated to membrane water flux via PIP1s.

In **Chapter 5** we wanted to show that what we found so far in the model species *Arabidopsis thaliana* with respect to the role of transpiration and water and sucrose translocation also applied to other and commercially more important cultivated crops. For this, we used the woody crop *Malus domestica* 'Gala', devoid of a root system, and attempted to ameliorate its growth *in vitro*. We studied growth in vessels with various gas replacement filters and found that apple microplant growth was best stimulated in the vessel with the highest lid permeability. We hypothesized that the decrease in humidity facilitated transpiration and herewith medium nutrient delivery and biomass accumulation. We investigated to what level leaf transpiration occurred *in vitro* and whether leaf transpiration was involved in nutrient transport *in vitro*. Acid fuchsine translocation in stems was followed as proxy of assimilated nutrients. Shoots transpiring more showed faster and more intensive staining indicating the correlation between transpiration and element flow *in vitro.* Next, a hygroscopic potassium chloride solution was placed inside a vial to reduce humidity and stimulate transpiration and thus increase

biomass. Microplants grown in potassium chloride-supplemented containers exhibited a rise in transpiration and an increase in dry weight. Subsequently, we explored whether manipulating transpiration levels might lead to improved plantlet growth in shoot cultures of *Malus domestica* 'Gala', We did so by interfering with stomatal and cuticular transpiration pathways. δ-Aminolevulinic acid was used to open stomata and was tested for its influence on growth. We found increased transpiration and biomass accumulation. We also investigated the effect of a herbicide, metolachlor, which is supposed to inhibit cuticle formation, on plantlet growth *in vitro.* Finally, we attempted to reduce adaxial cuticle formation by applying organic solvents and investigating effects on plantlet water loss, indicative for transpiration, and growth. However, the cuticle manipulation tests had an inhibitory or toxic effect on plantlets. Together, the results of this chapter demonstrated that it is possible to optimize plant growth *in vitro* through stimulation of transpiration, especially by manipulation of stomatal transpiration. Similar techniques might be applied in a general way for growth promotion of cultured microplants of other species *in vitro*.

In summary, our research described in this thesis demonstrated the existence of transpiration in conventional high humidity tissue culture vessels, pointed out its importance in contributing to plantlet growth in tissue culture, found the first clues for exogenous sucrose delivery in roots as mediated by sucrose transporters, revealed the influence of aquaporins PIP1s on growth and highlighted the increase of biomass in *Malus domestica* through manipulation of transpiration *in vitro*. In **Chapter 6** a description of the current status of the micropropagation/tissue culture industry is given and the results from this thesis are summarized and discussed. Suggestions for future studies are also provided.

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At almost the end of my PhD career I am like a centenarian man walking staggered up to a gate connecting an uneasy past and an unknown future. Suddenly a million thoughts and memories are crowding in my mind. It is a mixed feeling, real but unexpressive, distant but clear, as if I were walking along some track but now look back. It has been a long journey of life and finally I achieve a Doctor's title here in Wageningen University and Research. I would like to express my gratefulness to all who have done so much for me.

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About the author

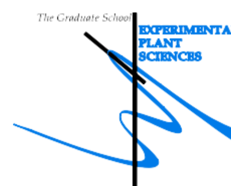
Curriculum Vitae

Huayi Li was born on February 11th 1988 in Xi'an City, Shaanxi Province, China. In 2008 he started his bachelor career in the field of biological engineering in the College of Innovation and Experimentation at Northwest Agriculture & Forestry University. After receiving his bachelor degree he continued his master study in the State Lab of Crop and Stress Biology for Arid Areas at Northwest A&F University. In 2014 he applied for a fellowship from the China Scholarship Council and continued his study as a PhD student at Wageningen University & Research. This thesis presents the outcome of his PhD research at Plant Breeding Wageningen U&R in the research group *Ornamentals, tissue culture & gene technology.*

Training and Education Statement

Education Statement of the graduate school

Experimental Plant sciences



Issued to: Huayi Li
Date: 08 April 2020
Group: Plant Breeding
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Nutrient flowing and explant growth in tissue culture	17 Nov 2015	1.5
► **Writing or rewriting a project proposal**		
Nutrient flow in in vitro propagated plants	2015-2016	4.5
► **Writing a review or book chapter**		
► **MSc courses**		
Plant Breeding 1 (PBR-22303)	Feb-Mar 2016	3.0
Plant Cell and Tissue Culture (PPH30306)	Mar-Apr 2016	3.0
Subtotal Start-Up Phase	12.0	

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD student days Get2Gether, Soest, NL	28-29 Jan 2016	0.6
EPS PhD student days Get2Gether, Soest, NL	9-10 Feb 2017	0.6
EPS PhD student days Get2Gether, Soest, NL	15-16 Feb 2018	0.6
► **EPS theme symposia**		
EPS Theme 1 Symposium Developmental Biology of Plants, Wageningen, NL	21 Jan 2016	0.3
EPS Theme 4 Symposium Genome Biology, Wageningen, NL	16 Dec 2016	0.3
EPS Theme 3 Symposium Metabolism and Adaptation, Wageningen, NL	14 Mar 2017	0.3
EPS Theme 1 Symposium Developmental Biology of Plants, Wageningen, NL	30 Jan 2018	0.3
EPS Theme 3 Symposium Metabolism and Adaptation, Wageningen, NL	13 Mar 2018	0.3
► **Lunteren Days and other national platforms**		
Annual Meeting Experimental Plant Sciences, Lunteren, NL	11-12 Apr 2016	0.6
Annual Meeting Experimental Plant Sciences, Lunteren, NL	10-11 Apr 2017	0.6
Annual Meeting Experimental Plant Sciences, Lunteren, NL	9 Apr 2018	0.3
Annual Meeting Experimental Plant Sciences, Lunteren, NL	8-9 Apr 2019	0.6
NVPW autumn symposium, Wageningen, NL	14 Dec 2018	0.3
NVPW spring symposium, Wageningen, NL	14 Jun 2018	0.3
► **Seminars (series), workshops and symposia**		
Symposium: Science - From Local to Global, Wageningen, NL	3 May 2017	0.3
Seminar: Candidate for the position of full professor Plant Physiology, Wageningen, NL	15 Sep 2017	0.1
Seminar: Prof.dr. Wyss - Highlights of hidden insect-worlds, Wageningen, NL	2 Oct 2017	0.1
Symposium: Preventing the end of the world - How science can save the planet, Wageningen, NL	2 Nov 2017	0.3
Symposium: Wageningen PhD Symposium - Bridging Science and Society: Unifying Knowledge, Wageningen, NL	17 May 2018	0.3
Symposium: Breeding Data: Statistical Advances in Modern Plant Breeding, Wageningen, NL	16 Oct 2018	0.3
Seminar: Rewiring starch metabolism for plant environmental adaptation, Wageningen, NL	1 Nov 2018	0.1
Seminar: Reimagining the future of high-throughput cell screening using the Beacon platform, Wageningen, NL	25 Apr 2019	0.1
► **Seminar plus**		
► **International symposia and congresses**		
The 10th European Plant Science Retreat, Utrecht, NL	3-6 Jul 2018	0.9
The 30th International Horticultural Congress, Istanbul, Turkey	12-16 Aug 2018	1.5
The 3rd International Conference “Plant Cells & Tissues In Vitro”, Vienna, Austria	1-2 Jul 2019	0.6
The 5th International Conference “Plant Transformation & Biotechnology”, Vienna, Austria	3-4 Jul 2019	0.6
► **Presentations**		
Poster: Transpiration and its role in in vitro growth of Malus domestica ‘Gala’ shoots, Annual Meeting Experimental Plant Sciences	9 Apr 2018	1.0
Poster: The growth increase of micropropagated plants in vitro through manipulation of stomatal transpiration, Wageningen PhD symposium	17 May 2018	1.0
Poster: Transpiration determines Arabidopsis growth in vitro. Role of stomata and cuticle, The 10th European Plant Science Retreat	5 Jul 2018	1.0
Talk: Transpiration and somatic embryo callus function, bijeenkomst van TKI-U project KV 1310-067	28 Jun 2017	1.0
Talk: Transpiration and growth of apple ’Gala’ micropropagation in vitro, bijeenkomst van TKI-U project KV 1310-067	27 Sep 2017	0.0
Talk: Stomata traits alteration effect on growth in vitro, bijeenkomst van TKI-U project KV 1310-067	10 Jan 2018	0.0
Talk: Transpiration and role of cuticle in vitro determining plant growth,bijeenkomst van TKI-U project KV 1310-067	12 Apr 2018	0.0
Talk: Search for sugar transporter genes involved in sugar translocation in Arabidopsis in vitro, bijeenkomst van TKI-U project KV 1310-067	12 Jul 2018	0.0
067	11 Oct 2018	1.0
Talk: How to reach societal relevance with your research, Wageningen PhD symposium	17 May 2018	1.0
Talk: Increased transpiration results in more growth in in vitro grown Malus shoots, II International Symposium on Micropropagation and In Vitro Techniques conference	13 Aug 2018	1.0
Talk: Plant tissue culture: nutrient flow SWEET genes mediate sugar translocation in roots of in vitro Arabidopsis plantlets for growth, NVPW autumn symposium	14 Dec 2018	1.0
Talk: The relation between transpiration and growth in in vitro micropropagation, exemplified in Malus domestica cv. ‘Gala’ plantlets, NVPW Spring symposium	14 Jun 2019	1.0
Talk: Plant tissue culture: the relation between transpiration and growth in in vitro micropropagation, the 3rd International Conference “Plant Cells & Tissues In Vitro”	2 Jul 2019	1.0
► **IAB interview**		

		date	cp
►	**Excursions**		
	Company visit at R&S Tissue Culture Laboratories	10 Sep 2015	0.3
	PhD company visit at KeyGene	12 Oct 2017	0.2
	PhD company visit at Dümmen Orange	15 Jun 2018	0.3
	PhD company visit at Koppert Biological Systems	26 Oct 2018	0.2
	Subtotal Scientific Exposure	22.2	

3) In-Depth Studies		*date*	*cp*
►	**Advanced scientific courses & workshops**		
	PhD course: Basic Statistics, Wageningen, NL	10-17 May 2017	1.5
	Summer course: Towards a Global One Health, Wageningen, NL	27-31 Aug 2018	1.5
►	**Journal club**		
►	**Individual research training**		
	Training for tissue culture at Euro-Tiss	1 Sep 2015	0.3
	Subtotal In-Depth Studies	3.3	

4) Personal Development		*date*	*cp*
►	**General skill training courses**		
	EPS Introduction Course, Wageningen, NL	11 Feb 2016	0.3
	Symposium: Interwoven - How science and art meet belowground, Wageningen, NL	14 Dec 2016	0.2
	PhD Workshop Carousel, Wageningen, NL	7 Apr 2017	0.3
	Competence Assessment, Wageningen, NL	19 Apr 2017	0.3
	Preparation Workshop for Career Day, Wageningen, NL	30 Jan 2018	0.1
	Career Day, Wageningen, NL	6 Feb 2018	0.3
	Course: Reviewing a Scientific Paper, Wageningen, NL	15 Mar 2018	0.1
	Course: The Final Touch: Writing the General Introduction and Discussion, Wageningen, NL	10 Apr 2018	0.6
	Course: Scientific Writing, Wageningen, NL	23 Apr-19 Jun 2018	1.8
	Course: Efficient Writing Strategies, Wageningen, NL	16 Apr-11 Jun 2018	1.3
►	**Organisation of meetings, PhD courses or outreach activities**		
►	**Membership of EPS PhD Council**		
	Subtotal Personal Development	5.3	

TOTAL NUMBER OF CREDIT POINTS*	**42.8**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	
** A credit represents a normative study load of 28 hours of study.*	

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