Physiological and molecular control of adventitious root formation in ornamental cuttings

Cumulative

Habilitation Thesis

to receive the *Venia Legendi*

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by

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Dedicated to

my parents Margot and Werner Drüge, a blacksmith and metalworker,
both who believed in and supported my potential since I was a young school boy

and

my wife Birgit, my faithful companion through life.

Preface

The present habilitation thesis is based on research activities, which were performed at the Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt (IGZ) if not indicated otherwise. The projects aimed at the elucidation of plant physiological factors and processes determining the adventitious root formation and leaf senescence in shoot tip cuttings of ornamental crops. In this context, the influences of specific environmental factors affecting the cuttings during the complex chain of global young plant production were considered as well as the principle influence of cutting excision involving the wounding and isolation of the cutting from the entire stock plant.

Fourteen publications resulting from the projects will be compiled and discussed in this thesis. The publications, which involved cooperation with other scientists in particular Dr. Roland Kadner, Dr. Siegfried Zerche and Prof. Dr. Philipp Franken from IGZ and include data from PhD studies of the former students Dr. Vijaya Rapaka, Dr. Yvonne Klopotek, Dr. María Ángeles Agulló-Antón, Dr. Amir Ahkami and Dr. Sandra Lischewski, were mainly conducted by the author or supervised by him as principle investigator, if not indicated otherwise. In publication (5), the author was responsible for the carbohydrate aspect of the study and shared responsibility for the whole study with the first author. In publication (9), the author shared the responsibility and supervision of the former PhD student Dr. Yvonne Klopotek with the last author. In publication (13), the author was responsible for the experiments focussing on the local and temporal distribution of auxin and shared responsibility for the whole study with Dr. Mohammad-Reza Hajirezaei at the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, where the experiments concerning the primary metabolism and histology of AR formation were performed. In publication (14), the author was mainly responsible for the array experiment, conducted the detailed analysis of the auxin- and ethylene-related transcriptome and developed the model. The included experiments on the function of ethylene biosynthesis and signalling were performed at the Leibniz Institute of Plant Biochemistry in Halle and there were supervised by Prof. Bettina Hause.

After a general introduction (1) into the subject of this thesis, the second part provides an overview (2) summarizing the publications as assigned to the following topics: 2.1 Influence of beneficial root-colonizing fungi at stock plant and cutting level on rooting of cuttings (publications 1-2), 2.2 Nitrogen and carbohydrate interactions on rooting of cuttings (publications 3-5), 2.3 Involvement of carbohydrate source and sink relations in rooting of cuttings (publications 6-11), 2.4 Involvement of plant hormone pathways in leaf senescence and adventitious root formation in cuttings (publications 12-14). This overview mainly considers the temporary context of knowledge when the publications were written. After the listing of the publications (3), the discussion part (4) integrates and critically discusses the obtained results in the contemporary context of science. General concepts are derived and an outlook on further needs of research is presented.

The publications, which have been compiled in chapter 3, will be cited in bold face letters. Additional publications of the author of this thesis will be cited with underlined characters.

Table of contents

Abbreviations

ABA	abscisic acid
ACC	aminocyclopropene-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AIL	aintegumenta-like
AI	intensity of adventitious root formation
AM	arbuscular mycorrhiza
AMF	arbuscular mycorrhizal fungi
AP2/ERF	apetala 2/ethylene response factor
AR	adventitious root
ARF	auxin response factor
AVG	aminoethoxyvinylglycine
AW	Adventivwurzel
AUX/LAX	auxin resistant/like auxin resistant permease, auxin influx transporter
Aux/IAA	type of auxin repressor
CF	chlorophyll fluorescence
Ci	*Chrysanthemum indicum*
CW	cell wall
Di	*Dianthus caryophyllus*
DLI	daily light integral
dpe	days post excision
dpin	days post insertion (planting)
EIN3	ethylene-insensitive 3
Ep	*Euphorbia pulcherrima*
ERF	ethylene responsive transcription factor
GFP	green fluorescent protein
GH3	Gretchen Hagen 3
Glc6PDH	glucose-6-phosphate dehydrogenase
GUS	ß-glucuronidase
hpe	hours post excision
hpin	hours post insertion (planting)
HXK	hexokinase
IAA	indole-3-acetic acid
IAA-AAH	IAA-amino acid hydrolase
INVcw	cell wall invertase
INVcyt	cytosolic invertase
INVvac	vacuolar invertase
JA	jasmonic acid
MCP	1-methylcyclopropene
N_t	total nitrogen content in dry matter
NPA	naphthylphthalamic acid
Om	organic substrate, modified
Pi	*Piriformospora indica*

PAT	polar auxin transport
Pe	*Pelargonium x hortorum*
PFK	phosphofructokinase
PH	plant hormones
PID	PINOID
PIN	pin-formed, auxin efflux transporter
P_N	net photosynthesis
Pt	*Petunia hybrida*
PPFD	photosynthetic photon flux density
QC	quiescent center
qN	non-photochemical quenching of chlorophyll fluorescence
QTL	quantitative trait loci
R_D	dark respiration
S	senescence
SAUR	small auxin up RNA
SN	silver nitrate
SnRK1	SNF1-related protein kinase
STS	silver-thiosulfate
T6P	trehalose-6-phosphate
TF	transcription factor
TIR/AFB	transport inhibitor response/auxin-signalling F-box
TLC	thin cell layer
TOR	target of rapamycin
TPL	TOPLESS, auxin and jasmonate co-repressor
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
WOX	wuschel-related homeobox
YFP	yellow fluorescent protein

1. Introduction

Adventitious root (AR) formation is a process in which new roots are formed post-embryonically in organs other than roots (Blakesley et al., 1991). This developmental process on the one hand discloses the fascinating regeneration potential and plasticity of plants allowing to withstand and to survive stress conditions. On the other hand, AR formation particularly in stem tissues of excised plant parts (= cuttings) is the crucial physiological process utilized for vegetative propagation of many horticultural plant species (Hartmann, 2011). The structures used in clonal propagation of many ornamental plant species are herbaceous shoot tip cuttings, which are generated by excision of young, mostly axillary shoots from stock (donor) plants and which consist of a leafy stem with the terminal shoot apex and at least one fully developed leaf. The principal demand on such cuttings is intensive AR formation within the shortest possible rooting period (Drüge, 1999) to restore "whole plant" functional integrity as prerequisite for subsequent survival, development and growth (Druege, 2009). Furthermore, rooting of each particular batch of cuttings should be synchronous among individual cuttings (Drüge, 1999) to avoid additional sorting and to provide uniform starting material for large scale production and increasing automation in horticultural industry (Druege, 2009).

Nowadays, the production of young ornamental plants via rooting of shoot tip cuttings is carried out on a global scale and involves a complex chain (Druege, 2009): (i) cutting production on stock plants, (ii) postharvest storage of cuttings, iii) transport of cuttings to rooting stations, and, finally (iv) the planting and cultivation for rooting. Cutting production is often carried out in low-latitude regions where high irradiance allows for rapid development of a high number of cuttings per stock plant but also causes high light adaptation of the photosynthetic apparatus (Forschner and Reuther, 1984). To synchronize cutting yields, which can exceed 1 million cuttings per day on a particular farm, with the frequently varying demand for specific cultivars and for the preparation of cuttings for subsequent transport, freshly harvested cuttings are immediately stored in darkness at low temperatures before shipment to the rooting stations, for example in Central Europe. Depending on particular plant species and mostly based on empirical data, temperature during storage is reduced to a minimum level that reduces catabolic processes in cuttings without causing severe cold stress or damage. However, whereas temperature can be controlled at optimum levels in cold chambers, subsequent air and ground transport can involve large temperature fluctuations (Kadner et al., 2000).

Insufficient root formation with certain plant species or cultivars impairs the producer's ability to bring plants to market. Furthermore, knowledge gaps concerning the influence and interactions of particular environmental factors impair optimum control of the production chain. This frequently causes losses in young-plant production, even for easy–to-root species. In 1999, De Klerk et al. (1999) calculated the amount of starting material produced via rooting of cuttings in the Netherlands to six billion plants per year and estimated the losses due to inadequate rooting to approximately 5% and 25% in ornamental and nursery crops, respectively. With certain ornamental crops such as *Pelargonium hortorum*, losses of individual batches of cuttings due to insufficient rooting can be much higher and particularly involve dark-induced leaf senescence and die off before roots are formed (Paton and Schwabe, 1987; Kadner et al., 2000; **Druege et al., 2004**; **Kadner and Druege, 2004**). This stays in contrast with the increasing demand for fast and synchronous AR formation to meet horticultural standards.

From a physiological point of view, a shoot tip cutting is an excised young shoot, which has experienced a) wounding and b) isolation from the network of signal- and resource fluxes of the donor plant (Druege, 2009). Thus, a certain degree of stress is inevitably linked to the cutting state. Considering the injury and isolation from the water- and nutrient-supplying root system of the stock plant, the first challenge for a cutting is to survive the postharvest phase. Even more, all leaves should remain in a vital state because they can be expected to have important source functions for AR formation and because decay of leaves causes additional cleaning or sorting of cuttings and particularly enhances the susceptibility to facultative parasitic fungi such as *Botrytis cinerea*.

In response to the excision, a new developmental program is initiated in specific plant cells to regenerate the lost root system. AR formation involves different successive phases and in stem tissues has repeatedly been observed to originate in or in close proximity to the interfascicular cambium adjacent to the vascular bundles (Blakesley et al., 1991; De Klerk et al., 1999). Even there is no consensus about the terminology and extension of different phases in literature, provided that AR formation does not start from preformed root primordia, the most common model distinguishes between three different phases, which are majorly based on anatomical criterions and responses to plant hormones (Jarvis, 1986; Kevers et al., 1997; De Klerk et al., 1999; Da Costa et al., 2013). The initial phase, mostly termed as ***induction phase***, is characterized as anatomical lag-phase, in which no cellular changes can be observed (Kevers et al., 1997; De Klerk et al., 1999). Considering the auxin response of apple microcuttings, De Klerk et al. (1999) even distinguished a very early phase from the induction phase and termed it ***dedifferentiation phase,*** postulating that during this phase the cells become competent for AR formation via obtaining auxin sensitivity. Considering this concept and also the response of AR formation to ethylene and gibberellic acid, Da Costa et al. (2013) recently distinguished between ***early*** and ***late induction phase***, while the early induction phase covers the dedifferentiation phase postulated by De Klerk et al. (1999). Following the induction phase, the ***initiation phase*** starts with first quality changes of cell structures e.g. swelling of nuclei and increasing density of cytoplasma, followed by cell division and subsequent differentiation of cell clusters into dome-shaped root primordia. The last, so-called ***expression phase*** starts with the differentiation of primordia into roots with differentiated vascular bundles connected to the vascular cylinder of the stem, followed by elongation and emergence of roots from the stem. For simplification, initiation and expression phase are sometimes joined under the same nomination ***formation phase*** (Fett-Neto et al., 1992).

AR formation in cuttings is influenced by a large set of environmental and diverse endogenous factors. Plant hormones have a particular role in reprogramming of cell fate and control of root tissue differentiation, with an outstanding role of auxin (De Klerk et al., 1999; Ludwig-Müller, 2009; Da Costa et al., 2013). The importance of cell reprogramming for AR formation in cuttings is reflected by strong regulation of gene transcription in the rooting zone (Brinker et al., 2004). However, the initiation and subsequent differentiation of ARs further depend on a sufficient supply of minerals, carbohydrates and other organic compounds to the rooting zone where they are used as resources to power and feed biosynthetic processes (Haissig, 1986; Blazich, 1988; Veierskov, 1988). In addition to the general abundance of such resources in the cutting, their distribution and transport between source and sink tissues should determine the intensity of AR formation (Druege, 2009). In this context, three successive metabolic phases have been

characterized for petunia cuttings, indicating that early establishment of a carbohydrate sink in the rooting zone is an important step (Ahkami et al., 2009).

The research presented in this habilitation thesis aimed at identification of physiological and molecular factors and processes controlling AR formation in shoot tip cuttings, while special attention was given to the influence of specific environmental factors that constitute key components of the global young plant production chain. In addition to AR formation, another focus was set on leaf senescence and resulting die-off of cuttings, when combinations of plant species and environmental conditions provoked such responses.

In the following chapter, an overview on the work is presented in four sections which cover different topics. In each section, the background, essential results and main conclusions of the considered publications are summarized in the temporary context of knowledge when the publications were written. Chapter 3 contains the references of the integrated scientific publications.

In chapter 4, the main results and conclusions presented in chapter 2 and 3 are integrated and discussed in the contemporary context of literature and general concepts are derived. At the end, the concepts are summarized in a postulated explanatory model on important physiological and molecular factors and processes regulating AR formation in cuttings. Important environmental factors during the young plant production chain and important functional units of shoot tip cuttings are considered. Finally, an outlook on future work is presented.

2. Overview

Figure 1 illustrates the influencing factors and the focus of analyses for the different plant species studied in the four topics of this chapter.

The first topic deals with the question, whether AR formation and survival of cuttings can be enhanced via inoculation of cuttings with arbuscular mycorrhizal fungi (AMF) at stock plant level and with *Piriformospora indica* at cutting level and which physiological principles may be involved.

Thereafter, the view will go deeper into the endogenous control of AR formation and the focus will be set on the contribution of initial nitrogen reserves in cuttings and initial carbohydrate levels in different cutting tissues to AR formation. The interaction between nitrogen pre-conditioning and cold dark storage of cuttings of three plant species will be considered.

The third part sheds light on the components and contribution of carbohydrate source and carbohydrate sink during AR formation in cuttings. Research on *Pelargonium*, *Dianthus* and *Petunia* will be presented regarding influences of pre-harvest light adaptation, dark storage, auxin application as well as light intensity, carbon dioxide and temperature during rooting. In these studies, analyses in terms of leaf senescence, histology and final intensity of AR formation, chlorophyll fluorescence, stomatal gas exchange, levels of metabolites, enzymatic activities and gene transcript abundance were applied.

In the last part, the involvement of plant hormone pathways will be addressed by analysis at metabolic and gene expression levels and involving pharmacological manipulation of hormone related pathways. At first, the focus is set on the role of ethylene perception in ethylene production and dark storage-induced leaf senescence of *Pelargonium* cuttings, which is a special problem particularly observed with this plant species. Thereafter, the involvement of polar auxin transport and transcriptional regulation of plant hormone pathways in adventitious formation of cuttings was investigated using *Petunia hybrida* as a model.

Some of the studies presented in this chapter refer to the conditions of photosynthetically active radiation (400-700nm) recorded at crop level (if not indicated otherwise) during cutting production (stock plant cultivation), storage of cuttings, or the cultivation of planted cuttings for rooting. To allow comparison between the studies, independent on the presentation of conditions in the original publications those light conditions are presented as daily light integral (DLI, mol m^{-2} d^{-1}) which integrates both the duration of daily photoperiod and the light intensity. In addition, the photosynthetic photon flux density (PPFD, µmol m^{-2} s^{-1}) is presented when the intensity of photosynthetically active radiation was controlled over constant length of photoperiod.

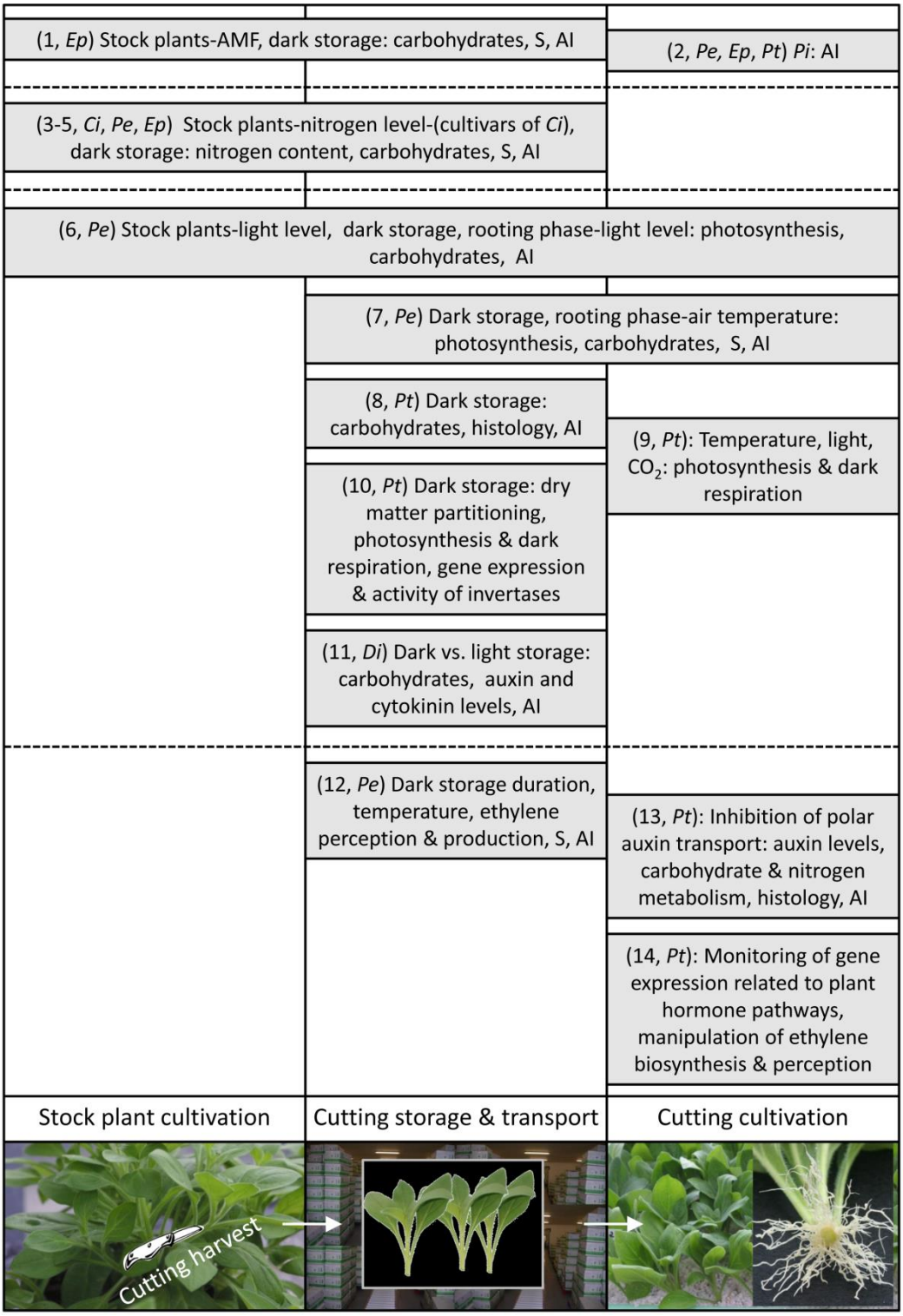

Figure 1: Scheme of the influencing factors studied as related to the phases of the young plant production chain for the different plant species (illustrated for *Petunia hybrida*) and the focus of analyses in the four topics (separated by dithered lines, topic 1 in the top). Between brackets: numbers of publications as they appear in chapter 3 and investigated plant species. S, senescence; AI, intensity of AR formation; AMF, arbuscular mycorrhizal fungi; *Pe*, *Pelargonium x hortorum*; *Ep*, *Euphorbia pulcherrima*; *Ci*, *Chrysanthemum indicum*; *Pt*, *Petunia hybrida*; *Di*, *Dianthus caryophyllus;* *Pi*, *Piriformospora indica*.

2.1 Influence of beneficial root-colonizing fungi at stock plant and cutting level on rooting of cuttings

When plants are growing in the soil their roots are mostly colonized by non-pathogenic microorganisms, which can increase plant resistance and/or tolerance to stresses and promote plant development and growth. In this context, the term endophyte is usually applied to organisms „which, for all or part of their live cycles, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease“ (Wilson, 1995). Focussing on fungi, endophytes are distinguished from mycorrhizal fungi, which colonize plant roots but grow into the rhizosphere. Considering that stock plants and harvested cuttings are usually planted in peat-based, mineralic or even sterilized substrates where presence of propagules of microorganisms may be extremely low (Azcon-Aguilar and Barea, 1997; Koltai, 2010), there might be a chance to improve clonal propagation by application of beneficial microorganisms. One aim of this thesis was to elucidate the potential of two different types of root-colonizing fungi to enhance survival and/or to stimulate AR formation in cuttings and to get indication, which physiological principles may be involved.

Arbuscular mycorrhiza (AM) is a mutualistic symbiosis occurring between root-colonizing fungi of the phylum *Glomeromycota* and circa 80% of plant species (Strack et al., 2003). While this association is characterized by a bidirectional transport of nutrients between symbionts – of carbohydrates from the plant to the fungus and of mineral nutrients, especially phosphate, and water from the fungus to the plant -, it has a great influence on overall plant physiology contributing to improved plant health and growth, particularly under suboptimal conditions (Peuss, 1958; Hirrel and Gerdemann, 1980; Sharma et al., 1992). This may involve improved uptake of nutrients (George, 2000) and water (Auge, 2001) and also increased carbon assimilation and export from leaves (Drüge and Schönbeck, 1993; Douds et al., 2000; Gernns et al., 2001). There are numerous studies indicating that survival of cuttings can be enhanced and AR formation can be stimulated when propagules of AM fungi are applied to the cuttings (**Druege et al., 2006** and references therein). However, rooting capacity of cuttings is determined already when cuttings are attached to the stock plant (Haissig, 1986). Considering the multifaceted influence of AM on plant physiology, we tested the hypothesis, that inoculation of stock plants with AM fungi can enhance subsequent survival and AR formation of harvested cuttings (**Druege et al., 2006**). To further evaluate the potential use of AM fungi in organic cultivation, stock plants of *Euphorbia pulcherrima* Willd. Ex Klotzsch were cultivated in three different substrates (one conventional: peat-based, two organic: peat-reduced) under non-limited nutrient conditions and half of plants were inoculated with *Glomus intraradices* (Smith and Schenk). Excised cuttings were either planted immediately for rooting or first dark stored at 9-10°C for 1 week. Independent of the cultivation system, mycorrhization of stock plants significantly reduced the high decay rate observed on stored cuttings when rooting occurred at low light levels (DLI: 3.6 mol m^{-2} d^{-1}). In addition and obviously independent of cutting survival, AM in stock plants increased the number of ARs formed by the stored cuttings. The AM-mediated increase in total root length per cutting was most pronounced when cuttings were produced in a particular organic system (Om) and experienced high light conditions during rooting (DLI: ≥ 7.5 mol m^{-2} d^{-1}). AM did not significantly affect N, P, or K concentrations in the cuttings. However, the strongest rooting response to AM observed for the Om-cuttings was associated with outstanding higher sugar levels in the leaves at the time of harvest and higher starch levels in the stem base (rooting

zone) after storage (**Druege et al., 2006**). It was concluded that mycorrhization of stock plants can reduce the risk of postharvest decay of excised cuttings and enhance AR formation after exposure to storage. Considering the influences of the light level on the rooting response to AM and the effects of AM on carbohydrate levels, the authors suggested that both higher carbohydrate reserves and a carbohydrate-reflected higher photosynthetic activity may contribute to the enhanced rooting of cuttings excised from mycorrhizal stock plants. Regarding the changed hormonal balance in AM plants (Drüge and Schönbeck, 1993; Ludwig-Müller, 2000), plant hormones particularly auxin and cytokinins were suggested to be also involved in the improved survival and rooting of cuttings derived from mycorrhizal stock plants (**Druege et al., 2006**).

AM fungi are obligate symbionts and can only be produced in living roots (Azcon-Aguilar and Barea, 1997). The basidiomycete *Piriformospora indica* was isolated from the rhizosphere of bushes in the Thar Desert in India and was characterized as endophyte, which colonizes plant roots but can be easily cultivated in axenic culture (Varma et al., 1999). Inoculations of a broad variety of plants with *P. indica* had positive effects on their biomass production, yield, flowering, and tolerance and resistance to abiotic and biotic stresses (**Druege et al., 2007** and references therein). Considering these effects mimicking those of AM fungi, it was hypothesized that inoculation of rooting substrate with *P. indica* should promote AR formation in cuttings (**Druege et al., 2007**). The effect of the endophyte on AR formation was tested on one cultivar per each of three species, respectively of *Pelargonium hortorum, E. pulcherrima* and *Petunia hybrida*. Inoculation with *P. indica* strongly enhanced the number and length of ARs formed in *P. hortorum* and *E. pulcherrima*. Comparing the strong enhancement of AR formation with the relatively low colonization of roots by the endophyte at the end of rooting period, it was suggested that *P. indica* acted on AR formation before physical contact to the plants was attained. *P. hybrida* showed a generally much faster rooting than the two other plant species, did not respond to the inoculation and also revealed lowest colonization rates with the endophyte. The authors suggested that the short time period of rapid root development may have not allowed effectual interaction with the fungus, or that levels of root promoting factors in the petunia tissues may have been above a certain level, which could not be further increased by the endophyte. Considering the strong dependency of AR formation on plant hormones and early recognition events detected in *Arabidopsis* seedlings during interaction with *P. indica* (Peskan-Berghofer et al., 2004), **Druege et al. (2007)** postulated that auxin, ethylene or other regulators might be involved in the stimulating effect of *P. indica* on AR formation.

2.2 Nitrogen and carbohydrate interactions on rooting of cuttings

Adequate nitrogen (N) supply to stock plants is an important concern for the propagation industry since N has a strong influence on both yield and quality of cuttings and new knowledge in this area is readily transferable to new fertilization strategies. Even though the principle importance of carbohydrate metabolism for AR formation in cuttings is out of question, the major bottlenecks at the levels of carbon assimilation, distribution and utilization are only poorly understood (Druege, 2009). N supply strongly influences carbohydrate distribution within plants by modifying processes of C assimilation, allocation and partitioning. Preliminary studies on the influences of nitrogen and carbohydrate levels in cuttings on AR formation in diverse plant species produced a large amount of conflicting data (**Druege et al., 2000** and references therein)

indicating that these relationships might dependent on plant genotype and also on other environmental factors. In this context, the important factors of dark storage of cuttings and of light acclimation of the photosynthetic apparatus to the light condition at the cutting production site (Forschner and Reuther, 1984) had been considered only marginally.

Setting the focus on the contribution of carbohydrates, the aims of the investigations described in this chapter were to

- analyse the interdependency of nitrogen content and carbohydrate levels in cuttings in the control of AR formation in *Chrysanthemum indicum*, *P. hortorum* and *E. pulcherrima*, considering the interaction of nitrogen level supplied to stock plants and dark storage of cuttings and influences of light conditions
- evaluate the contribution of carbohydrate pools in the different cutting parts, such as leaves and the stem base, to AR formation.

At first, we investigated the effects of three different doses of N (low, medium, high) supplied to stock plants of two cultivars of chrysanthemum (*C. indicum*), and of post-harvest cold dark storage of cuttings (0.5 C or 5 °C) for different periods. The responses of concentrations of total nitrogen content in dry matter (N_t) of excised cuttings and of carbohydrates in different cutting tissues at time of excision and after the different storage periods were analysed in relation to the number and length of subsequently formed ARs **(Druege et al., 2000**). Both cutting production on stock plants and cultivation of cuttings for rooting were conducted under high light conditions (DLI outside the greenhouse: 18 and 21 mol m^{-2} d^{-1}, respectively). Increasing the nitrogen supply to stock plants resulted in substantially lower starch levels in tissues of cuttings at time of excision and this effect was most pronounced in leaves. This was explained by an N-induced increase in the use of carbon skeletons for N assimilation limiting the accumulation of assimilated C in the form of starch (**Druege et al., 2000**). At the same time, sucrose levels in leaves were highest from stock plants grown at the highest N rate and there was a significant positive correlation between N_t and the sucrose:starch ratio in leaves of excised cuttings. Considering that a higher sucrose:starch ratio in leaves had been discussed as indicator of enhanced carbohydrate partitioning towards assimilate export (Galtier et al., 1993), it was suggested that a higher sink strength in the high nitrogen fertilized stock plants deriving from N assimilation contributes to the changed carbohydrate partitioning in high nitrogen stock plants which may also cause change fluxes of other co-transported compounds (**Druege et al., 2000**). Since a significant portion of nitrogen was supplied as ammonium nitrate in the study, the root-derived sink in particular was assumed to be involved in the changed carbohydrate partitioning. When cuttings were dark stored after excision, irrespectively of N rate, starch completely disappeared from leaves within two weeks and, to a large extent, from stems. By contrast, cuttings maintained higher sugar levels during four weeks of storage. This was particularly evident in low-nitrogen cuttings, whereas increased rate of N supply to stock plants and high N_t in cuttings at harvest promoted subsequent depletion of sugars during storage. The dark storage-induced depletion of carbohydrates was explained by energy- and carbon-consuming metabolic processes which were slowed down but not prevented at the low temperatures.

Irrespective of dark storage, and despite the fact that carbohydrate levels in cutting tissues were in most cases reduced with increasing N_t in the cuttings, number and length of formed ARs were positively correlated with N_t up to highest levels of 6.8% of dry matter. Thus, nitrogen content at

time of cutting excision provided a primary bottleneck for AR formation. This was explained by the important function of N as component of diverse organic compounds such as amino acids which may limit root initiation and further development (**Druege et al., 2000**). However, rooting of both cultivars was under the influence of nitrogen and dark storage positively correlated also with the sucrose:starch ratio in leaves at time of cutting excision. Considering these relationships, it was suggested that the carbohydrate export from leaves manifested at harvest in response to the nitrogen supply to stock plants was causally involved in the enhanced AR formation of nitrogen-rich cuttings **(Druege et al., 2000**). Under the experimental conditions, the lower carbohydrate reserves in the high-nitrogen cuttings particularly found after storage did not impede subsequent AR formation to such an extent that the positive effect of nitrogen was significantly inhibited. By contrast, the finding that stored cuttings particularly of one cultivar produced longer roots than their non-stored counterparts documents a benefit of storage to AR formation in chrysanthemum. Considering that chrysanthemum cuttings were shown to have a high photosynthetic activity, it was suggested that under high light conditions the carbohydrate demand of AR formation is covered by current photosynthesis (**Druege et al., 2000**).

Other than chrysanthemum, cuttings of pelargonium (*P. hortorum*) are sensitive to leaf senescence and had been described to reveal a weak photosynthetic activity during rooting particularly after being adapted to the high light levels prevailing in low-latitude regions of stock plant cultivation (Forschner and Reuther, 1984). Furthermore, since finished plants are demanded between April and May, the cuttings have to cope with low light conditions during the winter rooting period. Taking this young plant production chain challenges into account, we investigated how the rate of N supplied to pelargonium stock plants and subsequent dark storage (7d, 10 °C) of cuttings influenced carbohydrate partitioning, cutting survival and AR formation **(Druege et al., 2004**). Stock plants of the cv. 'Isabell' were grown under high light supply to simulate light conditions of a low latitude cutting production site (DLI: 16.2 mol m^{-2} d^{-1}) and the harvested high-light-adapted cuttings were rooted at low light (DLI: 1.3 mol m^{-2} d^{-1}) to approximate the average natural light conditions prevailing in Central European greenhouses between November and February. Changes in carbohydrate levels in the stems and leaves of cuttings to N and storage were very similar to results described and discussed for chrysanthemum cuttings (**Druege et al., 2000**). Thus, the increasing rate of N supply decreased starch levels in tissues of harvested cuttings particularly in leaves and promoted subsequent depletion of sugars during storage. It was discussed that the strong response of leaf starch to dark storage may also be linked to its transitory character being mobilized in source leaves under darkness during the diurnal cycle and also during low light conditions (Geiger et al., 2000). The reduced depletion of sugars in the low-nitrogen cuttings may result from the initially higher starch levels or from lower carbon utilization during the storage period possibly resulting from lower carbohydrate demand for N assimilation (**Druege et al., 2000, 2004**). Interestingly, further monitoring of carbohydrate levels during the rooting period revealed that sugars accumulated in the leaf lamina of previously stored cuttings, whereas carbohydrates were exhausted simultaneously in all other cutting parts including the leaf petioles and the stem base. **Druege et al. (2004)** suggested that cold dark storage impaired subsequent export of carbohydrates from the leaves, possibly via disturbed phloem loading since the symplasmic route of sucrose transport seems to be particularly susceptible to low temperatures (Van Bel, 1993).

The number of ARs formed in the unstored cuttings was positively correlated with N_t in whole cuttings at harvest (**Druege et al., 2004**) as was the case with chrysanthemum (**Druege et al., 2000**). However, with pelargonium this relationship disappeared when cuttings were cold dark stored. By contrast to N_t and irrespective of dark storage, initial sugar levels in cutting tissues before planting (determined at time of excision for unstored cuttings and after the storage period for the stored cuttings) were positively correlated with the percentage of cuttings which survived. Furthermore, the number of ARs formed by the survived cuttings depended on initial leaf sugar levels, while best relationships were found for glucose. Interestingly, the sugar concentration in the leaves showed a much stronger relationship to AR formation than carbohydrate concentrations in the stem base and in the whole cutting (mg per g dry matter of cutting) or the carbohydrate content in whole cuttings (mg per cutting). The positive relationships between initial sugar levels in leaves and intensity of subsequent AR formation were confirmed in a sample survey with cuttings of 21 pelargonium cultivars provided from different production sites at low latitudes (**Druege et al., 2004**).

The limiting role of initial leaf sugars was highlighted by the kinetics of carbohydrates in the stem base during the process of root formation. Thus, the sugar level in the leaves at the time of planting determined the level of sugars achieved in the stem base at day 5 after planting (dpin = days post planting), and this intermediate level in the stem base was positively correlated with the final number of ARs (**Druege et al., 2004**). It was concluded that AR formation in pelargonium can be limited by the initial amount of N reserves in the cutting. However, this relationship is superimposed by a predominant effect of carbohydrate availability that depends on initial carbohydrate levels at time of planting, when high-light adaptation and low current light conditions impair net carbon assimilation. Then, the initial sugar levels in cutting leaves are determining the subsequent export and abundance of carbohydrates in the stem base there limiting the development of ARs.

Contrasting to pelargonium but similar to chrysanthemum, cuttings of *E. pulcherrima* are rooted mainly during the summer under sufficient light. The interaction between nitrogen conditioning of cuttings and dark storage of this species were unknown when we included this plant species in our conception and studied the interrelationships between nitrogen, carbohydrate levels and rooting of the cv. 'Cortez Red' (**Zerche and Druege, 2009**). After being harvested from stock plants which received three different levels of nitrogen supply, cuttings were either planted immediately or first dark stored at 9-10 °C for 1 week. To get indication on the involvement of light level during rooting, the last of three cutting harvests was rooted in October under significant lower light in the greenhouse (DLI: 3.3 - 3.9 µmol m^{-2} d^{-1}) compared to the other harvests in September (DLI: 5.8 - 6.3 µmol m^{-2} d^{-1}) and August (DLI: 7.2 - 7.7 µmol m^{-2} d^{-1}). Carbohydrates were analysed in fully developed leaves (oldest and second oldest leaf) and in the stem base of cuttings of the last two harvests. Nitrogen and dark storage affected the carbohydrate levels in same ways as found for similar tissues in chrysanthemum **(Druege et al., 2000**) and pelargonium (**Druege et al., 2004**)**.** According to chrysanthemum rooted under sufficient light, number and length of ARs formed by *E. pulcherrima* were positively correlated with N_t in whole cuttings at time of excision. This relationship was irrespective whether cuttings experienced storage or not. It was even observed when the overall rooting intensity was reduced under the lower natural light during October (**Zerche and Druege, 2009**). However, independently on N_t, all rooting parameters of the two harvests in September and October were

positively correlated also with the level of sugars in the fully developed leaves at time of planting. Multivariate regressions accounting for N_t at harvest, leaf sucrose level at time of planting and DLI during the rooting period explained 74% and 79% of total variances in root number and root length per cutting, respectively. It was concluded that N content in cuttings is a dominant factor determining the rooting capacity of *E. pulcherrima* particularly when rooting occurs under sufficient light, as is commonly available during propagation of this plant species **(Zerche and Druege, 2009**). However, considering the enhanced AR formation found with higher initial leaf sucrose levels and higher light integral during the rooting period and the finding that dark storage of cuttings did not impair rooting when cuttings experienced high light in August, **Zerche and Druege (2009**) suggested a second limitation of AR formation in *E. pulcherrima* by carbohydrate export from leaves being subject to an interaction of initial carbohydrate levels and current photosynthesis.

2.3 Involvement of carbohydrate source and sink relations in rooting of cuttings

2.3.1 Interaction of carbohydrate reserves and photosynthesis

Taking into account the high sensitivity of AR formation in high-light adapted pelargonium to storage-induced carbohydrate depletion when rooting occurs under low light (**Druege et al., 2004**), we further analysed the interplay between initial carbohydrate reserves, current photosynthesis and AR formation in cuttings under the influences of light abundance at stock plant and cutting level and dark storage of cuttings **(Rapaka et al., 2005**). We focussed on the following questions:

- How do light levels during stock plant growth and during rooting of cuttings influence photosynthesis of leaves and carbohydrate levels in the different tissues?
- What are the contributions of initial carbohydrate status *versus* current photosynthesis to the subsequent carbohydrate dynamic in cuttings during rooting and to AR formation?
- Can measurements of initial carbohydrate concentrations and knowledge of current photosynthetic activity be integrated into a common theory of how carbohydrates limit rooting?

To answer these questions, stock plants of the cv. 'Isabell' were grown at different levels of photosynthetic light per day (achieved by winter-, spring-, and summer production in a glass house, DLI ranging from 2.6 to 11.5 mol m^{-2} d^{-1}) and rooted at different light levels, either in a shaded greenhouse during the different seasons (DLI ranging from 1.9 to 6.0 mol m^{-2} d^{-1}) or in a climate chamber at a DLI of 3.2 mol m^{-2} d^{-1}. To minimize effects of leaf senescence, cuttings were exposed to a short storage for 4 days at variable low temperatures to simulate a transport situation. Different parameters of modulated chlorophyll fluorescence (CF) as well as the partitioning of individual carbohydrates in leaf lamina and the basal portion of the cutting stems were analysed before and during AR formation and related to the number of formed ARs.

Increasing the light level supplied to the stock plants raised carbohydrate levels in the stem base of cuttings at time of excision but not in the leaves. **Rapaka et al. (2005)** suggested that stock plants exported the surplus C assimilated at the higher irradiance to active sinks, thereby accumulating C in the young stem tissues. In contrast, higher light exposure of stock plants

reduced the mean non-photochemical quenching (qN) of CF in cuttings during subsequent rooting under low light. Considering theories on relationships between quenching parameters of CF and photosynthetic processes, **Rapaka et al. (2005)** postulated that the photosynthetic apparatus of cuttings was less prepared to cope with lower irradiance after adaptation to higher irradiance. The lower qN of high-light adapted cuttings was assumed to reflect a depressed proton gradient across the thylakoid membrane, resulting in a decreased synthesis of ATP. When unstored cuttings produced under different light levels were rooted under constant low light (DLI: 3.2 mol m^{-2} d^{-1}), the sucrose concentration in leaves at day 7 post planting (dpin) was dependent on qN, and thus highest in the low-light adapted cuttings. Furthermore, under these conditions higher sucrose levels accumulated at 7 dpin and more ARs were finally formed in the stem base of low-light adapted cuttings compared to high-light adapted cuttings, while the number of ARs was positively correlated with qN. It was concluded that low-light adaptation of cuttings at stock plant level improved AR formation under low light via enhancing low light photosynthetic efficiency generating more sucrose in source leaves to be transported to the stem base (**Rapaka et al., 2005**).

The short-term storage of cuttings resulted in complete loss of leaf starch and depletion of leaf sugars, particularly of sucrose. However, concentrations of all sugars in leaves of stored cuttings after one week of rooting accumulated to same levels as measured in the unstored cuttings, which reflected the contribution of current photosynthesis to the carbohydrate balance in cuttings (**Rapaka et al., 2005**). Under the influence of dark storage, the accumulation of sugars in the stem base until 7 dpin was primarily dependent on the initial sugar reserves at time of planting and the light exposure during rooting. Under low-light rooting conditions (DLI 1.9 mol m^{-2} d^{-1}), similar to the previous study (**Druege et al., 2004**) the level of sugars accumulated in the stem base at 7 dpin was lower for the previously dark stored cuttings and was positively correlated to the initial sugar concentration in leaves at time of planting. Higher light during rooting enhanced the qN of CF in cutting leaves and eliminated the carbohydrate differences between unstored and stored cuttings. Storage of cuttings reduced AR formation only when the DLI during rooting was ≤ 3.2 mol m^{-2} d^{-1}. Under these conditions, the number of ARs was positively correlated with initial concentrations of leaf sugars, particularly of sucrose, whereas no consistent relationship was found with the initial concentrations sugars in the stem base. A higher light level during rooting counter-balanced the negative influence of storage on AR formation. When stored cuttings were rooted in high light conditions, intensity of rooting was apparently dependent on current photosynthesis as reflected by the positive correlation between the mean qN during rooting and number of roots.

The whole variation of AR formation in the experiment could be predicted by using the mean value of leaf sucrose concentrations between 0 and 7 dpin, accounting for both the initial and the 1-week equilibrium sucrose status of leaves. These results provided evidence that the limitation of AR formation in leafy cuttings by initial carbohydrate reserves is subject to the function of photosynthesis during rooting (**Rapaka et al., 2005**). Additionally, photosynthetic efficiency during rooting seems to be influenced by the light level during both stock plant production and during rooting. The data, as a whole, stress the importance of a high and steady export capacity of carbohydrates from the source leaves during rooting as major requirement for a sufficient influx of carbohydrates and possibly other co-delivered substances in the stem base to support high intensive AR formation (**Rapaka et al., 2005**).

When plants are grown with a sufficient amount of light, leaf net photosynthesis usually decreases with a reduction in temperature below an optimum level. This relationship between temperature and photosynthesis is usually weaker or even reversed under low light conditions when respiratory CO_2 production becomes more important than gross photosynthesis (Berry and Björkman, 1980). Considering these relationships and the low photosynthetic activity of pelargonium cuttings when rooting under low light (Forschner and Reuther, 1984; **Druege et al., 2004**; **Rapaka et al., 2005**), **Druege and Kadner (2008)** hypothesized that lower air temperatures during rooting could increase availability of carbohydrates and thereby improve post-storage survival and rooting of cuttings, provided that the temperature in the rooting zone is sufficiently high for root development. To test this hypothesis, stock plants of two pelargonium cultivars were adapted to high light conditions during growth (DLI: 8.7 mol m^{-2} d^{-1}) and cuttings taken from plants were stored in darkness for 4 d at 10 °C to lower carbohydrate reserves. After storage, cuttings were rooted in climate chambers in low light (DLI: 3.6 mol m^{-2} d^{-1}, 10 h photoperiod) with a root zone temperature of 20 °C, and air temperature of either 10 °C or 20 °C. The relative humidity was adjusted to 100% and 85% for day and night, respectively.

Lowering air temperature during rooting of pelargonium increased carbohydrate concentrations in leaves and in the stem base of cuttings during the rooting period when compared with the air temperature of 20 °C. In accordance to these findings, the same treatment enhanced the net photosynthetic rates of oldest leaves determined by stomatal gas exchange and also the production of shoot dry matter during the rooting period. Based on these results, **Druege and Kadner (2008)** concluded that the decrease in air temperature raised the C gain of cuttings, improved the replenishment of carbohydrate pools after storage-induced depletion, and possibly reduced respiratory C losses. When the stored cuttings were rooted at 20 °C their leaves became chlorotic and some cuttings decayed, particularly with cuttings from the cultivar 'Telemann'. Higher carbohydrate concentrations in cutting tissues during the rooting at low air temperatures were correlated with lower percentage of damaged leaf area. Considering suppressive effects of sugars at physiological concentrations on dark-induced leaf senescence in *Arabidopsis thaliana* (Chung et al., 1997), **Druege and Kadner (2008)** suggested that the higher carbohydrate levels repressed leaf senescence in pelargonium cuttings.

In addition to reduced leaf senescence, higher sugar levels during low temperature rooting were correlated with increased intensity of AR formation at the end of rooting period while best relationship were found for sucrose, particularly in leaves. It was concluded that with general low photosynthetic activity of pelargonium cuttings under low light, low air temperature-mediated increases in carbohydrates, particularly in leaf sugar pools, promote the export of sucrose to the stem base, thereby advancing the formation of ARs under the influence of the high root zone temperature (**Druege and Kadner, 2008**).

2.3.2 Interaction of carbohydrate source and carbohydrate sink in adventitious root formation in cuttings

The studies on pelargonium had highlighted the importance of carbohydrate source strength as important bottleneck for intensive AR formation in cuttings, particularly when dark storage of cuttings reduce carbohydrate reserves and photosynthetic activity of cuttings is low (**Druege et al., 2004**; **Rapaka et al., 2005**; **Druege and Kadner, 2008**). Because the genus *Petunia* provides

the structure of a shoot tip cutting and also has important qualities to serve as model plant for studying plant development (Gerats and Vandenbussche, 2005), we established *P. hybrida* as model to investigate AR formation in shoot tip cuttings in terms of anatomy, gene expression, enzymatic activities and primary metabolism (Ahkami et al., 2009). Monitoring of metabolites and activities of enzymes of primary metabolism indicated that the establishment of the new sink in the rooting zone (stem base) is an early metabolic key event during AR formation under normal light conditions (Ahkami et al., 2009). Based on these findings, the carbohydrate sink aspect was integrated into the conceptions of the studies which are summarized in this chapter.

Longer ARs formed in chrysanthemum after cold dark storage compared with immediately planted cuttings (**Druege et al., 2000**) indicated stimulating principles of dark storage on AR formation which can come into front when a carbohydrate deficit in cuttings is avoided.

Considering that the rooting response of cuttings to dark storage is obviously dependent on plant genotype (Garrido et al., 1998; **Druege et al., 2000**; **Druege et al., 2004**) and regarding the qualities of *P. hybrida* as model for analysis of AR formation (Ahkami et al., 2009), we studied the response of AR formation in *P. hybrida* cv. 'Mitchell' to dark exposure comparing different durations and temperatures (**Klopotek et al., 2010**). Dark exposure for seven days at 10 °C prior to planting accelerated AR formation. Thus, the time period needed for intensive AR formation under diurnal light (PPFD: 100 µmol m^{-2} s^{-1} per 10h photoperiod, DLI: 3.6 mol m^{-2} d^{-1}) was strongly reduced. When dark exposed and freshly excised cuttings experienced the same light period after planting, dark exposed cuttings produced a significantly higher number and length of ARs. Increase of temperature from 10°C to 20 °C during dark exposure caused a similar enhancement of AR formation but leaf damage and unusual fine roots were observed with the higher temperature. **Klopotek et al. (2010)** concluded that darkness is the primary factor stimulating AR formation in petunia which is most effective in combination with the lower temperature preventing exhaustion of cuttings.

To get indication on the dynamic of AR formation and on the involvement of carbohydrate metabolism during the dark exposure at 10 °C and upon subsequent transfer to diurnal light condition, anatomical stages of AR development were analysed and levels of carbohydrates were monitored in fully developed leaves and in the stem base. Root meristem formation already started during the cold dark exposure so that at day 7 post excision (dpe) 60% of cuttings had produced first meristematic cells (meristemoids). Furthermore, when the dark exposed cuttings were planted and exposed to light, the subsequent development of meristemoids to root meristems and primordia occurred much faster compared with cuttings, which received light from the beginning (**Klopotek et al., 2010**). During the dark exposure, levels of starch and sugars significantly decreased and this was more pronounced in fully developed leaves than in the stem base. This depletion, however, was compensated during the first 3 days after planting. At the same time after planting, previously dark exposed cuttings accumulated higher carbohydrate levels in the stem base than cuttings which were planted immediately after excision. Considering these relationships and also that first root meristemoids were already formed under darkness, **Klopotek et al. (2010)** suggested that the early phases of root development, induction and initiation, are probably less dependent on high abundance of carbohydrates. However, with regard to the dynamic of carbohydrates it was further suggested that dark exposure promoted subsequent accumulation and translocation of carbohydrates to the stem base under subsequent light, thereby enhancing the availability of carbon and energy needed for primordia development

and growth of ARs. It was pointed out, that this may involve a sugar depletion-induced higher photosynthetic rate enhancing overall carbohydrate availability in cuttings and/or an enhanced allocation of carbon to the developing roots, possibly driven by the new meristematic cells already formed during the dark phase (**Klopotek et al., 2010**).

Considering the obviously high contribution of carbon assimilation to the carbohydrate balance in *P. hybrida* **(Klopotek et al., 2010**), in the next study we analysed the level of net photosynthesis (P_N) and dark respiration (R_D) during the course of AR formation in freshly excised cuttings of the cv. 'Mitchell' (**Klopotek et al., 2012**). We also studied the response of the CO_2 gas exchange of cuttings to short-term variations in PPFD, CO_2 concentration and temperature, and its long-term acclimation to PPFD over a period of 14 dpin. To avoid artefacts possibly resulting from changes of the cuttings environment, a multiple open chamber system was established for measuring the CO_2 gas exchange of whole petunia cuttings using covered rooting trays as measuring cuvettes connected to an infrared CO_2 sensor. When cuttings were exposed to a PPFD of 100 µmol $m^{-2}s^{-1}$ per 10h photoperiod according to **Klopotek et al. (2010)**, shoot dry matter increased substantially during the first week already, when root growth was still absent. This indicates a strong carbon sink activity of the shoot in freshly excised cuttings **(Klopotek et al., 2012**). P_N of cuttings acclimated to these conditions was substantially high at same PPFD and further enhanced with short-term increases of PPFD up to a light saturation point at 376 µmol $m^{-2}s^{-1}$. Further, an almost linear increase of P_N was recorded when CO_2 concentration was temporarily increased from 300 until 1300 ppm. By contrast, increase in temperature from 14 °C up to 31 °C had only marginal effects on P_N, whereas R_D was strongly enhanced. Continuously higher P_N and R_D were recorded when cuttings were permanently exposed to a PPFD of 150 µmol $m^{-2}s^{-1}$ compared to 80 µmol $m^{-2}s^{-1}$ after 2 dpin, without showing variations during the process of AR formation. Furthermore, exposure of stock plants, which had been acclimated to a PPFD of 300 µmol $m^{-2}s^{-1}$ (DLI: 10.8 mol m^{-2} d^{-1}) before, to a PPFD of 100 µmol $m^{-2}s^{-1}$ (DLI: 3.6 mol m^{-2} d^{-1}) after one week significantly reduced the maximum P_N of fully developed source leaves, while P_N at 100 µmol $m^{-2}s^{-1}$ was on a level similar as determined for cuttings under same light conditions. It was concluded that under light conditions, which are typical for young plant production in Central Europe and were also applied by **Klopotek et al. (2010)**, petunia cuttings show high carbon assimilation from the first day after planting onwards. The data also indicates that CO_2 gas exchange in petunia is not affected by AR formation but subject to stock plant light acclimation and to environmental conditions prevailing during the rooting period.

Based on the findings that cold dark exposure promoted AR formation and that this was associated with increased levels of carbohydrates particularly in the stem base after the dark phase compared with immediately planted cuttings (**Klopotek et al., 2010**), we addressed the question, how carbohydrate metabolism could be involved in dark-stimulated AR formation in petunia (**Klopotek et al., 2016**). Taking into account that the observed carbohydrate depletion during the dark phase may enhance photosynthesis via feed-forward control, we firstly investigated, whether dark exposure increases subsequent net photosynthesis during the rooting under light, using the newly established open chamber measuring system (**Klopotek et al., 2012**). However, P_N and R_D were found on same levels for control and dark pre-exposed cuttings while P_N was similarly as high as determined for source leaves of shoots still attached to the stock plants of the same cultivar (**Klopotek et al., 2012**). It was concluded that P_N provides a substantial input of organic carbon into the cutting, being not altered by dark pre-exposure (**Klopotek et al.,**

2016). The data reflect a high robustness of the photosynthetic machinery in *P. hybrida* 'Mitchell', while the photosynthetic activity is obviously not limited by feed-forward control under the applied conditions. Based on these results, we followed the hypothesis that the enhanced AR formation in dark treated cuttings is the outcome of selectively higher carbon allocation towards the developing root system in the stem base. Therefore, we monitored the dry matter production of shoots versus roots over different periods. When freshly excised and previously dark exposed cuttings were planted and exposed to same light conditions, the dark treated cuttings allocated a higher proportion of dry matter to the rooting zone at the expense of reduced dry matter production in the shoot. It was concluded that dark exposure enhances the sink strength of the rooting zone against the upper shoot (**Klopotek et al., 2016**).

Invertases are important molecular drivers of sink strength, since they reduce the sucrose pool by converting it into glucose and fructose, which are further channeled into the metabolic pathways, and thereby regulate utilization and storage of organic carbon (**Klopotek et al., 2016**). Among the compartment-specific types of vacuolar invertases (INVvac), cytosolic invertases (INVcyt) and cell wall invertases (INVcw), the latter are considered to have an outstanding role in sink activity via modifying phloem unloading particularly in those tissues that undergo cell division and elongation (Lemoine et al., 2013). In our initial study on the involvement of primary metabolism during AR formation in *P. hybrida* under diurnal light, an early rise in INVcw activity was found in the stem base prior to first anatomical signs of AR formation and was suggested to contribute to sink establishment in the rooting zone (Ahkami et al., 2009). Considering these relationships, we monitored invertase activities and transcript levels of invertase-encoding genes in the stem base and in the shoot apex under the influence of dark exposure (**Klopotek et al., 2016**). It was assumed that the shoot apex is the most important utilization sink in shoot tip cuttings competing for carbohydrates with the rooting zone. Under darkness, higher activities of INVcyt and INVvac were maintained in both tissues when compared to cuttings under light. Further, darkness reduced the ratio of apex- to stem base-located RNA accumulation of one INVcyt and two INVvac genes which indicated preferential expression in the rooting zone. **Klopotek et al. (2016)** suggested that these genes and corresponding enzymes may have particular functions for the carbohydrate sink in specific tissues of the stem base under darkness. However, the activity of INVcw showed a rooting-zone specific strong increase under both diurnal light and dark conditions. Furthermore, the early response pattern of activity showed a subdued but significant rise under darkness compared to light conditions and this was mirrored by the RNA accumulation of the invertase gene *PhINVcw2*. It was suggested that the rooting zone specific early rise in expression particularly of *PhINVcw2* and the corresponding increase in INVcw activity contribute to early sink establishment occurring already during the dark period. Based on the results, a model on the involvement of carbohydrate source-sink relationships in the dark stimulation of AR formation in petunia cuttings was postulated in which rooting zone expression and activation of invertases have key functions (Figure 2).

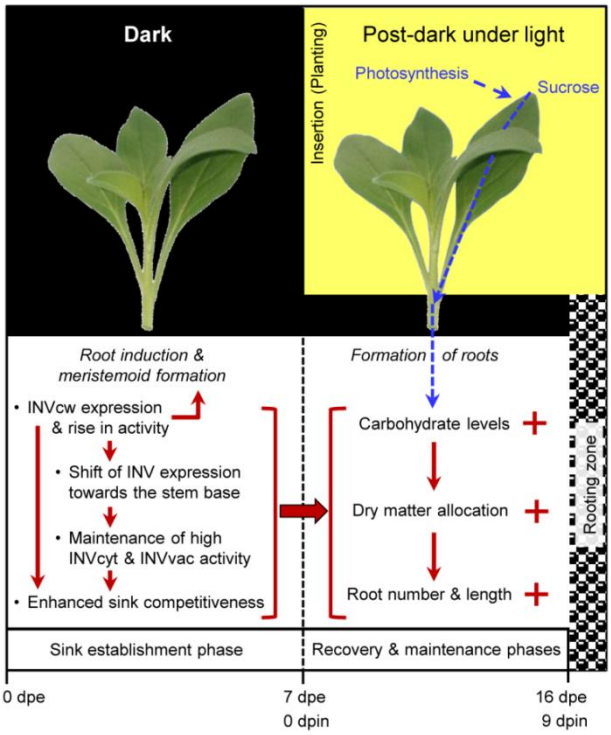

Figure 2. Schematic presentation of the involvement of carbohydrate source-sink relationships in the dark stimulation of AR formation in petunia cuttings, based on the current study and on carbohydrate and rooting data published in **Klopotek et al. (2010)**. Red arrows indicate stimulation. Red plus signs indicate promotive effects of dark pre-exposure compared to planting after excision **(Klopotek et al., 2016**).

A shift of expression of genes encoding specific INVcyts and INVvacs towards the stem base and a stem base specific expression of INVcw genes during darkness are postulated to enhance the sink competitiveness of the rooting zone against the shoot apex. When dark pre-exposed cuttings are planted and exposed to light, current photosynthesis provides synthesis of assimilates which are preferentially channelled to the rooting zone when compared with immediately planted cuttings. Wounding, other stresses resulting from the isolation of cuttings from the cutting, and changes in plant hormone homeostasis were discussed as candidate factors regulating the expression and activity of invertases (**Klopotek et al., 2016**).

Similar to *P. hybrida*, AR formation in cuttings of carnation (*Dianthus caryophyllus*) could be enhanced by cold dark storage of cuttings (Garrido et al., 1998). Because the auxin response of carnation cuttings was changed after storage, the authors assumed that auxin is involved in the promotive effect of storage. Regarding the general role of auxin as effective inductor of AR formation and findings on other plant species, that application of auxin stimulated the translocation of assimilates towards the rooting zone, we aimed to evaluate the carbohydrate source versus carbohydrate sink limitation of AR formation in carnation under the influence of cold storage considering interrelationships with auxin (**Agulló-Antón et al., 2011** and references therein). The magnitude of carbohydrate source was modulated by application of continuous low light (PPFD: 10-15 µmol $m^{-2}s^{-1}$ per 24 h, DLI: 1.1 mol m^{-2} d^{-1}) during cold storage compared to continuous darkness and to immediate planting of cuttings and exposure to light. Carbohydrate levels in fully developed leaves and the stem base were monitored and concentrations of the cytokinin zeatin and of indole-3-acetic acid (IAA) were measured at the end of the storage period.

After planting, the number and lengths of formed ARs under diurnal light (PPFD: 100 µmol $m^{-2}s^{-1}$ per 10 h photoperiod, DLI: 3.6 mol m^{-2} d^{-1}) were determined, also in dependence on auxin application. Dark storage for up to 4 weeks increased the percentage of early rooted cuttings and the final number and length of ARs, despite decreased sugar levels in the stem base after the storage period. Application of low light during storage greatly enhanced the sugar levels, particularly in the stem base and there also decreased IAA levels and increased the zeatin/IAA ratio.

However, the same treatment inhibited subsequent AR formation compared to cuttings which experienced continuous darkness during cold storage. Application of auxin to non-stored cuttings before planting enhanced the accumulation of sugars in the stem base. Auxin stimulated AR formation most strongly in non-stored, less so in light-stored, and only marginally in dark-stored cuttings (**Agulló-Antón et al., 2011**). Based on the results and further considering the key role of cytokinin-auxin crosstalk in controlling AR formation and meristem activity (Ludwig-Müller, 2009; Moubayidin et al., 2009) and that sugar application and enhanced sugar sensing suppressed auxin response in *Arabidopsis* in terms of lateral root growth and gene expression (Karve and Moore, 2009; Mishra et al., 2009), the authors postulated a model of auxin-sugar-cytokinin interactions in AR formation in carnation (Figure 3).

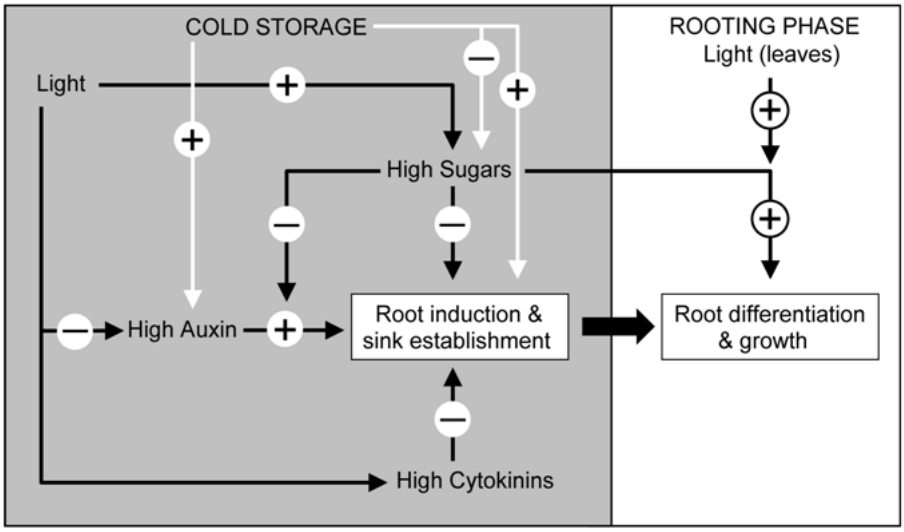

Figure 3. Schematic presentation of proposed interactions of auxins, cytokinins and sugars on AR formation in carnation cuttings under the influence of cold storage. Arrows marked alternatively with a plus or minus sign indicate promotional versus inhibitory influences, respectively (**Agulló-Antón et al., 2011**).

Cold storage of cuttings was suggested to bring forward AR induction and sink establishment in the rooting zone, both of which are assumed to be promoted by accumulation of auxin (early sink limitation of AR formation). Light exposure of cuttings during storage reduces IAA levels in the stem base as a result of direct light-mediated auxin oxidation, altered auxin transport or changed metabolism, and there also increases sugar and cytokinin concentrations to reach supra-optimum levels inhibiting AR induction. By contrast, post-storage light application to the planted cutting is expected to drive photosynthesis feeding the carbon demand of root differentiation and growth (source limitation of AR formation) (**Agulló-Antón et al., 2011**).

2.4 Involvement of plant hormone pathways in leaf senescence and adventitious root formation in cuttings

2.4.1 Involvement of ethylene perception in dark storage-mediated leaf senescence and adventitious root formation in pelargonium

Depending on the cultivar, cuttings of pelargonium (*P. hortorum*) may be highly susceptible to excision responding with postharvest leaf senescence. This response is strongly enhanced by dark storage of cuttings before planting, while the leaf senescence usually develops after planting during the rooting period (**Kadner and Druege, 2004**). Considering the strong impact of injury on ethylene production and the potential role of ethylene as trigger of senescence (Mattoo and White, 1991), a preliminary study of Kadner et al. (2000) focused on the dynamic of ethylene production of cuttings after excision under different temperature regimes. This study revealed an early ethylene production, which started immediately after cutting excision, peaked between 4 and 12 hours post excision (hpe) and returned to low levels at 24 hpe, when cuttings experienced a temperature of 15 °C. Exposure of cuttings to low temperature of 3 °C immediately after excision strongly inhibited ethylene production. However, when cuttings were rewarmed to 15 °C thereafter, a high ethylene production was detected. The amount of ethylene produced after the low temperature was significantly higher compared to cuttings, which were continuously exposed to the high temperature. This indicated that reduction of temperature blocks current production of wound-induced ethylene but induces production of additional chilling-induced ethylene, which is built on the top of the wound ethylene during the subsequent warm phase (Kadner et al., 2000).

In the next investigation, we analysed the contribution of ethylene action to dark storage-mediated leaf senescence in pelargonium, and studied in particular the role of ethylene action in ethylene production of cuttings when stored at different temperatures **(Kadner and Druege, 2004**). Comparing two cultivars with different sensitivity to cutting excision and dark storage, cuttings were treated with 1-methylcyclopropene (MCP), silver-thiosulfate (STS), or silver nitrate (SN) as inhibitors of ethylene reception, with ethylene or remained chemically untreated when stored at different temperatures. Both, ethylene production by cuttings during storage and post-storage senescence in terms of leaf damage and total drop-out rate were much higher when cuttings were stored at 20 °C compared to 12°C or 5 °C. The senescence recorded for the sensitive cultivar was higher compared to the tolerant cultivar and not changed by ethylene application while similar amounts of ethylene were produced by both cultivars. STS and SN application caused leaf damage indicating toxicity. Treatments with MCP reduced dark-storage induced senescence by circa 50% in both cultivars particularly at the higher storage temperatures but also reduced the intensity of AR formation. However, treatments with MCP and also with the other blockers strongly enhanced the ethylene production particularly at higher temperatures, while the levels reached in the containers indicated a non-attenuated production over the storage time. It was concluded that ethylene action partially accounts for dark storage-induced leaf senescence in pelargonium cuttings but that also other ethylene-unrelated processes are involved (**Kadner and Druege, 2004**). Considering further that ethylene application enhanced AR formation, ethylene seems to act also as a positive signal in AR formation in pelargonium. Furthermore, the strong enhancement of ethylene concentration after blocking ethylene perception, together with the transient character of ethylene production during continuous

measurement in the flow-through system under normal conditions (Kadner et al., 2000) indicate an negative feedback loop of ethylene production in the vegetative tissues of the cuttings, which was interrupted by the ethylene blocking (**Kadner and Druege, 2004**)**.**

2.4.2 Involvement of polar auxin transport and transcriptional regulation of plant hormone pathways in adventitious formation of cuttings

It is widely accepted that auxins, mainly IAA is an effective inducer of AR formation, whereas high auxin levels obviously have an inhibitory role at later stages (De Klerk et al., 1999; Ludwig-Müller, 2009). Though experimental data support the view that excision-induced AR formation in cuttings is dependent on polar auxin transport (PAT) and involves early accumulation of IAA in the rooting zone (Blazkova et al., 1997; Tonon et al., 2001; Garrido et al., 2002), a coherent picture on the functional relationship between PAT, auxin accumulation, and induction of AR formation was lacking until recently. We used *P. hybrida* cv. 'Mitchell' to analyse the relationships between plant hormones and excision-induced AR formation in cuttings. At first, we analysed the distribution of IAA in cuttings at time of excision and investigated the role of PAT in the temporal distribution of IAA in the stem base and relationships to carbohydrate metabolism and AR formation **(Ahkami et al., 2013**). A negative correlation was found between IAA concentration in leaves of different age and leaf fresh weight. This stays in accordance with corresponding relationships found for *Arabidopsis* where IAA levels were high in those leaves that showed high rates of cell division but strongly decreased when leaf expansion was initiated (Ljung et al., 2001). However, the IAA distribution within oldest leaves revealed higher concentration in the petioles compared to the adjacent leaf base. This suggests that active auxin transport occurred between the leaf and the stem of the petunia cutting at the time of cutting excision (**Ahkami et al., 2013**). Petunia cuttings excised and planted for rooting under diurnal light exhibited a double peak of IAA in the stem base between 2 and 24 hpe. The time course of IAA was mirrored by the expression pattern of the auxin responsive *GH3* gene following the IAA peak with a lag phase of ca. 2h. However, blocking of PAT with naphthylphthalamic acid (NPA), an effective inhibitor of auxin efflux (Morris et al., 2010), completely prevented the 24-hpe peak of IAA and severely inhibited the root meristemoid formation at 72 hpe and the final rooting of cuttings determined at 14 dpe. Furthermore, NPA treatment reduced the activities of INVcw and INVvac, which are considered as molecular drivers of sink activity in the newly developing sink of the rooting zone (Ahkami et al., 2009; **Klopotek et al., 2016**), during the period until 48 hpe. The same treatment also enhanced sugar levels and inhibited the rise of activities of phosphofructokinase and glucose-6-phosphate dehydrogenase, which control sugar entry into glycolysis and the pentose phosphate pathway, during the late phase of AR formation, and reduced levels of specific amino acids in a phase-specific manner. Considering these results, **Ahkami et al. (2013**) postulated a model of relationship between PAT, auxin accumulation, primary metabolism and AR formation in *Petunia* cuttings in response to excision from the donor plant, with a key role of PAT (Figure 4).

After excision of cuttings, PAT enables the accumulation of free auxin in the rooting zone, where it (1) contributes to the establishment of the new sink via stimulation of INVcw and INVvac and (2) induces the first cell divisions of the new root meristems. Activity of sucrose degrading enzymes provides the hexoses needed for cell proliferation, differentiation and growth while sugars may also have signal functions in these processes. After the decrease of auxin levels, which may be partially controlled by conjugation of IAA to amino acids catalysed by GH3

proteins, subsequent development of ARs stimulates the channelling of glucose into synthesis of amino acids, proteins and nucleic acids.

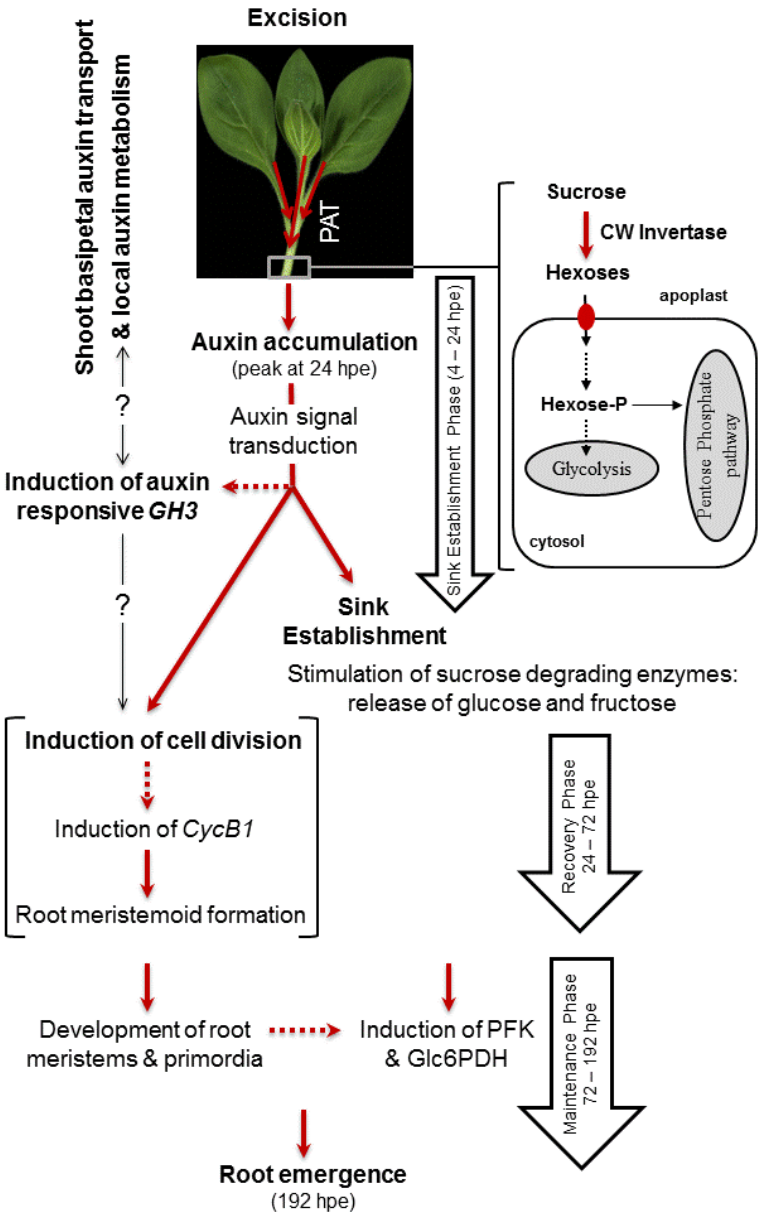

Figure 4: A postulated model of relationship between PAT, auxin accumulation, primary metabolism and AR formation in petunia cuttings in response to excision from the donor plant. Red solid arrows indicate processes that are dependent on PAT and are correlated with the resulting auxin peak at 24 hpe in the rooting zone. Red dashed arrows indicate additional processes which are hypothetically depending on PAT as supported by data of **Ahkami et al.** (2009, **2013**). Thin black arrows with question mark show possible interrelations between *GH3* induction, auxin metabolism and cellular events of AR formation. PAT: polar auxin transport, CW invertase: cell wall invertase, PFK: phosphofructokinase, Glc6PDH: Glucose-6-phosphate dehydrogenase, hpe: hours post excision (**Ahkami et al., 2013**).

In the next approach, we used a microarray to analyse the transcriptome of plant hormone-related genes in the stem base during excision-induced AR formation (**Druege et al., 2004**). The data disclosed multifaceted changes of the auxin transport system, auxin conjugation and the auxin signal perception machinery. Complementing the finding that the IAA-peak at 24 hpe was highly dependent on PAT (**Ahkami et al., 2013**), the transcriptome did not reveal any indication for excision-induced up-regulation of genes involved in auxin biosynthesis. However, early up-regulation of two isogenes of IAA-amino acid hydrolase (IAA-AAH), being also wound-inducible in leaves, may contribute to the very early NPA-insensitive IAA peak at 2 hpe (**Ahkami et al., 2013**) via hydrolysis of amino acid conjugates, whereas the subsequent repression of the same genes may contribute to the observed reduction of IAA level during the root formation phase. Seven out of 11 genes encoding proteins of the GH3 family showed a prolonged induction, while 6 transcripts exhibited a strong induction at 2 hpe already. One major function of GH3 proteins is their activity as IAA-amido synthetases, which are important for maintaining auxin homeostasis via conjugation of IAA to amino acids (Staswick et al., 2005). The data confirmed the findings of **Ahkami et al. (2013)** and suggest that shortly after excision the auxin metabolism is shifted towards long-term reduction of the IAA pool (**Druege et al., 2014**). However, considering the role of a particular GH3 protein in de-etiolation induced AR formation in intact hypocotyls of *Arabidopsis* (Gutierrez et al., 2012), some of the upregulated *GH3* genes in petunia may have other functions beyond conjugation of IAA (**Druege et al., 2014**)**.**

Even though AR formation in petunia shows a strong dependency on PAT (**Ahkami et al., 2013**), the microarray data did not indicate a general stimulation of the auxin transport machinery but rather points to a phase-specific fine-tuning of the system at transcriptional level (**Druege et al., 2014**). One gene for a putative efflux transporter was up-regulated from 2 hpe onwards and reached a 20-fold increase at 24 hpe when one PIN-like gene for an auxin transport protein was induced too. Thus, up-regulation of these efflux carrier genes was discussed to be involved in the observed PAT-dependent auxin peak at 24 hpe and PAT-dependent induction of AR formation (**Ahkami et al., 2013**; **Druege et al., 2014**). However, **Druege et al. (2014)** pointed out that even when auxin biosynthesis, metabolism and transport would remain unchanged, auxin should accumulate after excision of cuttings, because separation from the basal part of the plant should interrupt further basipetal transport from the stem base. Contrasting to the genes encoding putative auxin efflux carriers, two out of six influx carrier genes were down-regulated during the induction phase, before one of them was continuously up-regulated during the root formation phase. **Druege et al. (2014)** suggested a preferential role of auxin influx carriers during the formation of new meristems and subsequent differentiation. This stays in line with the proposed function of AUX/LAX controlled, acropetal auxin flux to the root apex as important factor controlling embryonic and lateral root development (Swarup et al., 2001; Peer et al., 2011). In addition, the petunia array data indicates differential expression of the protein kinase PINOID targeting PINs to the apical plasma membrane (Fozard et al., 2013) and of PINOID-binding proteins which modify PINOID activity (Benjamins et al., 2003). This suggests a further fine-tuning of the auxin transport machinery via intracellular localization of carriers (**Druege et al., 2014**).

The control of AR formation at the level of auxin signalling is poorly understood. A current model on the involved signalling cascade is based on studies on primary and lateral root development and on other developmental processes (Da Costa et al., 2013). Important regulating components in the nucleus are the TIR/AFB complex (transport inhibitor response/auxin-signalling F-box) and

the repressor proteins Aux/IAA. The latter recruit the co-repressor TPL (TOPLESS), being also a co-repressor in jasmonate signalling, to exert their repressive function on ARFs (auxin response factors) (Chapman and Estelle, 2009; Perez and Goossens, 2013). IAA cross-links the TIR1/AFB complex and the repressor proteins Aux/IAA. This allows ubiquitination and proteosomal degradation of the repressor Aux/IAA, releasing the ARFs from repression. The released ARFs act as activators or repressors on the transcription of auxin-responsive genes. In petunia cuttings, many genes putatively encoding ubiquitin-ligases of the TIR/AFB complex and ARFs were down-regulated shortly after excision, whereas up-regulation was only rarely observed (**Druege et al., 2014**). It was suggested that this response reflects a transcriptomic response toward overall reduction of auxin sensitivity after cutting excision, which may be based on a negative feedback (Benjamins and Scheres, 2008) to the early rise in IAA level. Because certain ARFs may act as repressors of AR formation (Gutierrez et al., 2012), the observed early down-regulation of certain ARFs may have contributed to the induction of AR formation (**Druege et al., 2014**). However, genes putatively coding for Aux/IAA proteins showed a strong regulation and the most phase-specific shifts in expression, which were mainly pronounced between 6 and 72 hpe. ARFs and Aux/IAAs have already been considered as important “auxin codes” for reprogramming phases of other developmental processes (Teale et al., 2006). The strong temporal variation in Aux/IAA expression supports the view, that these are important selective controllers of auxin response pathways during the specific phases of AR formation (**Druege et al.**, **2014**). Because the expression of Aux/IAA proteins is highly sensitive to auxin (Benjamins and Scheres, 2008) and also provides a linkage to other plant hormones (Brenner et al., 2005; Song et al., 2009; Cakir et al., 2013), the transcriptional regulation are expected to reflect both the changes in IAA level and crosstalk to other hormones during AR formation (**Druege et al., 2014**).

Contrasting to genes related to the auxin pathway, genes involved in ethylene biosynthesis and action showed a more uniform pattern as a high number of respective genes were generally induced during the whole process of AR formation. The transcriptome data suggest a strong excision-induced stimulation of ethylene biosynthesis at the levels of ACC (aminocyclopropane-1-carboxylic acid) synthesis and oxidation. While a substantially high number of genes of ACC synthase (ACS) and ACC oxidase (ACO) were also induced in leaves within 2 hours after wounding, most of the genes were continuously up-regulated during AR formation in the stem base. However, the time points of maximal induction indicated different principles of stimulation (**Druege et al., 2014**). The important role of ethylene for stimulating AR formation was demonstrated by the application of inhibitors of ethylene biosynthesis and perception as well as of the precursor ACC, all changing the number and length of ARs.

A model was proposed showing the putative roles of PAT and resulting auxin accumulation in initiation of subsequent changes in auxin homeostasis and signal perception and of wound- and stress-induced ethylene production and signalling as stimulating factors of AR formation (**Druege et al., 2014**).

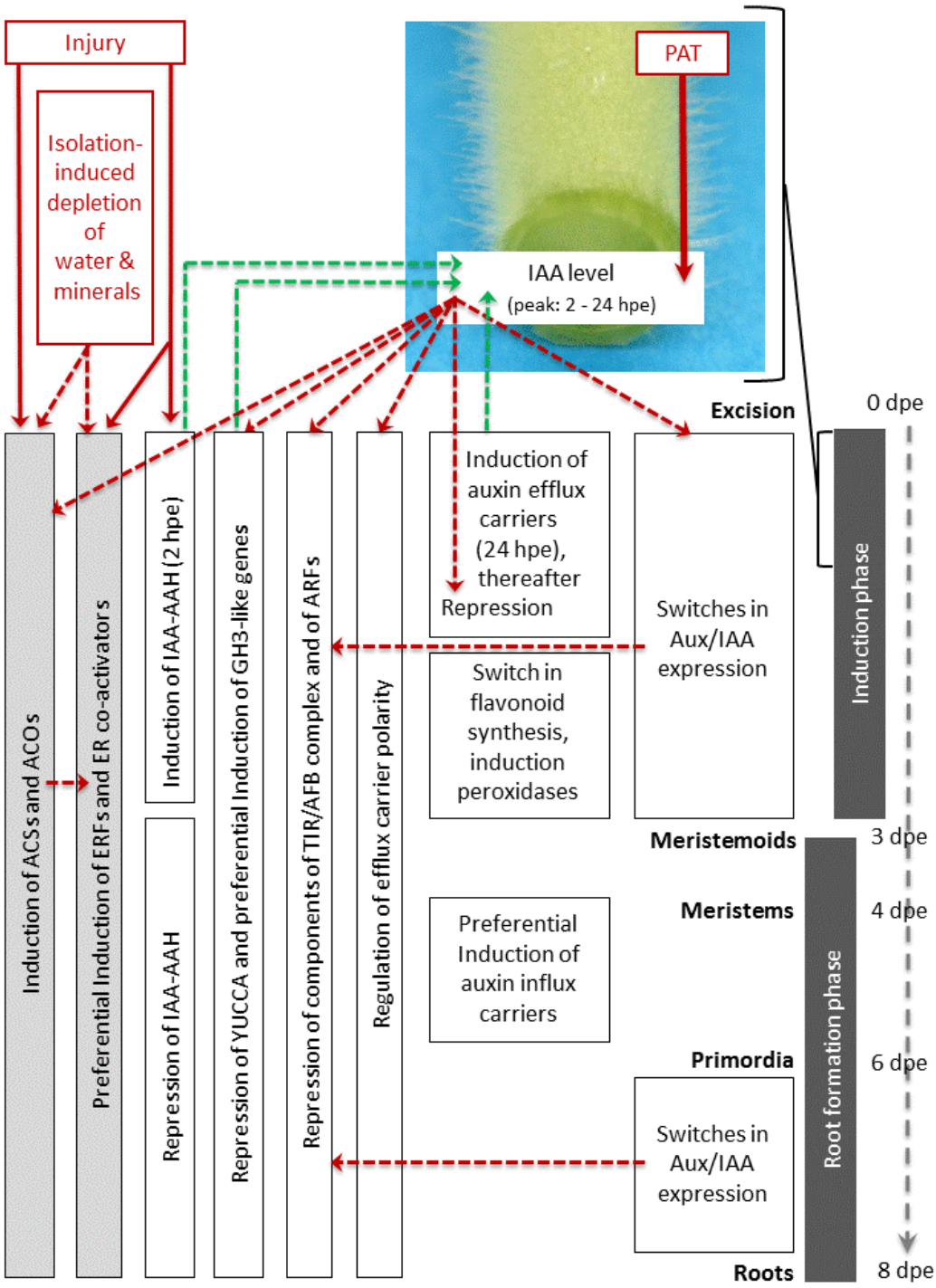

Figure 5: Postulated model of regulation of ethylene and auxin biosynthesis, of auxin transport and of ethylene and auxin signal perception at transcriptome level during AR formation in petunia cuttings. Red lettering marks important stimulating factors. Red arrows indicate the evident influence of PAT on IAA accumulation based on the results of **Ahkami et al. (2013)** and of injury on expression of *ACSs, ACOs*, ethylene responsive transcription factors (*ERFs)* and *IAA-AAH*. Red dashed arrows indicate hypothetically involved controls of gene expression based on the literature. Green dashed arrows indicate hypothetic links from gene expression to IAA levels based on the literature (**Druege et al., 2014**).

Cutting excision was suggested as initial event leading to an overflow of auxin because the basipetal auxin drain is interrupted. Using feedback loops to auxin transport and to auxin signalling, this auxin accumulation was proposed to stimulate a shift of the transcriptome (a) providing a buffering against the auxin overflow and to re-establish auxin homeostasis and (b) adjusting auxin signalling for subsequent initiation, differentiation and growth of new roots. Observed shifts in expression of genes controlling the flavonoid pathway between 24 and 72 hpe may modify auxin transport for example via interaction with efflux transporters, while the pronounced increase in the expression of peroxidases from 72 hpe onwards may further contribute to the decline of IAA after 24 hpe and maintenance of low levels thereafter. Ethylene biosynthesis, which is stimulated by wounding and does probably also respond to other stresses and auxin, was proposed to act as important stimulator of AR formation probably via the expression of ethylene responsive transcription factor genes, whereas the timing of different phases seems to be controlled by auxin. Functional analysis of candidate genes by generating transgenic lines through sense or anti-sense approaches and by use of reporter constructs was suggested as important approach for the future to determine the roles in the different phases of AR formation and to assign the different functional activities to particular tissues or cells (**Druege et al., 2014**).

3. List of scientific publications integrated in this thesis

Topic 2.1 Influence of beneficial root-colonizing fungi at stock plant and cutting level on rooting of cuttings

(1) **Druege, U**., Xylaender, M., Zerche, S., and von Alten, H. (2006). Rooting and vitality of poinsettia cuttings was increased by arbuscular mycorrhiza in the donor plants. Mycorrhiza 17, 67-72. https://doi.org/10.1007/s00572-006-0074-5

(2) **Druege, U**., Baltruschat, H., and Franken, P. (2007). Piriformospora indica promotes adventitious root formation in cuttings. Scientia Horticulturae 112, 422-426. https://doi.org/10.1016/j.scienta.2007.01.018

Topic 2.2 Nitrogen and carbohydrate interactions on rooting of cuttings

(3) **Druege, U**., Zerche, S., Kadner, R., and Ernst, M. (2000). Relation between nitrogen status, carbohydrate distribution and subsequent rooting of chrysanthemum cuttings as affected by pre-harvest nitrogen supply and cold-storage. Annals of Botany 85, 687-701. https://doi.org/10.1006/anbo.2000.1132

(4) **Druege, U**., Zerche, S., and Kadner, R. (2004). Nitrogen- and storage-affected carbohydrate partitioning in high-light-adapted Pelargonium cuttings in relation to survival and adventitious root formation under low light. Annals of Botany 94, 831-842. https://doi.org/10.1093/aob/mch210

(5) Zerche, S., and **Druege, U**. (2009). Nitrogen content determines adventitious rooting in Euphorbia pulcherrima under adequate light independently of pre-rooting carbohydrate depletion of cuttings. Scientia Horticulturae 121, 340-347. https://doi.org/10.1016/j.scienta.2009.02.012

Topic 2.3 Involvement of carbohydrate source and sink relations in rooting of cuttings

(6) Rapaka, V.K., Bessler, B., Schreiner, M., and **Druege, U**. (2005). Interplay between initial carbohydrate availability, current photosynthesis and adventitious root formation in Pelargonium cuttings. Plant Science 168, 1547-1560. https://doi.org/10.1016/j.plantsci.2005.02.006

(7) **Druege, U**., and Kadner, R. (2008). Response of post-storage carbohydrate levels in pelargonium cuttings to reduced air temperature during rooting and the relationship with leaf senescence and adventitious root formation. Postharvest Biology and Technology 47, 126-135. https://doi.org/10.1016/j.postharvbio.2007.06.008

(8) Klopotek, Y., Haensch, K.-T., Hause, B., Hajirezaei, M.-R., and **Druege, U**. (2010). Dark exposure of petunia cuttings strongly improves adventitious root formation and enhances carbohydrate availability during rooting in the light. Journal of Plant Physiology 167, 547-554. https://doi.org/10.1016/j.jplph.2009.11.008

(9) Klopotek, Y., George, E., **Druege, U**., and Klaering, H.-P. (2012). Carbon assimilation of petunia cuttings in a non-disturbed rooting environment: Response to environmental key factors and adventitious root formation. Scientia Horticulturae 145, 118-126. https://doi.org/10.1016/j.scienta.2012.08.004

(10) Klopotek, Y., Franken, P., Klaering, H.-P., Fischer, K., Hause, B., Hajirezaei, M.R., and **Druege, U**. (2016). A higher sink competitiveness of the rooting zone and invertases are involved in dark stimulation of adventitious root formation in *Petunia hybrida* cuttings. Plant Science 243, 10-22. https://doi.org/10.1016/j.plantsci.2015.11.001

Topic 2.4 Involvement of plant hormone pathways in leaf senescence and adventitious root formation in cuttings

(11) Agulló-Antón, M.A., Sanchez-Bravo, J., Acosta, M., and **Druege, U**. (2011). Auxins or sugars: what makes the difference in the adventitious rooting of stored carnation cuttings? Journal of Plant Growth Regulation 30, 100-113. https://doi.org/10.1007/s00344-010-9174-8

(12) Kadner, R., and **Druege, U**. (2004). Role of ethylene action in ethylene production and poststorage leaf senescence and survival of pelargonium cuttings. Plant Growth Regulation 43, 187-196. https://doi.org/10.1023/B:GROW.0000045999.61765.7e

(13) Ahkami, A.H., Melzer, M., Ghaffari, M.R., Pollmann, S., Javid, M.G., Shahinnia, F., Hajirezaei, M.R., and **Druege, U**. (2013). Distribution of indole-3-acetic acid in Petunia hybrida shoot tip cuttings and relationship between auxin transport, carbohydrate metabolism and adventitious root formation. Planta 238, 499-517. https://doi.org/10.1007/s00425-013-1907-z

(14) **Druege, U**., Franken, P., Lischewski, S., Ahkami, A.H., Zerche, S., Hause, B., and Hajirezaei, M.R. (2014). Transcriptomic analysis reveals ethylene as stimulator and auxin as regulator of adventitious root formation in petunia cuttings. Frontiers in Plant Science 5:494. https://doi.org/10.3389/fpls.2014.00494

4. Discussion and Outlook

In this chapter, the findings summarized in chapter 2 will be discussed also in the context of current concepts on the role of plant hormones in regulation of AR formation and leaf senescence. Auxin will be considered as important inductor of AR formation and master regulator in hormonal crosstalk during this process (Da Costa et al., 2013; Pacurar et al., 2014). Ethylene will be considered as important positive regulator in leaf senescence (Kim et al., 2015). With regard to AR formation, ethylene and cytokinins mainly act as inhibitors of AR induction but can have promotive influences during the early induction (dedifferentiation) while ethylene may additionally stimulate the expression of ARs (Da Costa et al., 2013). There is intensive auxin-ethylene and cytokinin-auxin crosstalk in plants (Bishopp et al., 2011; Da Costa et al., 2013; Marhavy et al., 2014). Recent studies on pea cuttings and intact hypocotyls of *Arabidopsis* support the concept that strigolactones have a suppressive function in AR formation and that inhibition of PAT is involved (Rasmussen et al., 2012). Since both cytokinins and strigolactones are mainly produced in the root system, the lack of root-derived cytokinins and strigolactones is considered to contribute to AR formation in cuttings (Steffens and Rasmussen, 2016).

4.1 Influence of beneficial fungi at stock plant and cutting level

Concept 1: Beneficial effects of arbuscular mycorrhiza and P. indica on rooting of cuttings indicate involvement of carbohydrate metabolism and plant hormones.

4.1.1 Pre-conditioning of cuttings by arbuscular mycorrhiza

In our study, enhanced survival and AR formation of cuttings harvested from AM-colonized stock plants was obviously related to carbohydrate metabolism and dependent on light conditions (**Druege et al., 2006**). This suggests that the acclimation of shoot carbohydrate metabolism to the mycorrhiza-derived sink in the root system can provide a higher capacity in the shoot to power and feed the development of new roots, also after the mycorrhizal root system has been removed. However, the implementation of this capacity obviously depends on light conditions allowing for sufficient photosynthesis of the cuttings (**Druege et al., 2006**).

Whereas no detailed studies on carbohydrate metabolism in mycorrhizal stock plants and excised cuttings are available in literature, investigations of the systemic metabolic response of diverse host plants to infection with AMF highlight the mycorrhiza-induced complex change of shoot carbohydrate metabolism. Thus, AM plants have repeatedly been shown to reveal higher carbon assimilation of leaves, which is often associated with increased opening of stomata (Boldt et al., 2011 and references therein; Schweiger et al., 2014). In *Plantago major*, higher carbon assimilation was associated with a strong shift of the foliar metabolome which was to a great extent unrelated to the P content of the leaf (Schweiger et al., 2014). Comparison of the response of sugar levels in leaves with those in roots to mycorrhizal infection indicated that a high proportion of the enhanced carbon gain is transported to the root system (Boldt et al., 2011; Doidy et al., 2012). Sugar transporters have been identified, which are up-regulated in the shoots of mycorrhizal plants (Garcia-Rodriguez et al., 2005; Boldt et al., 2011; Doidy et al., 2012). These results and those of carbon allocation studies with ^{13}C labelling (Olsson et al., 2010) indicate that a fine-tuned control at different levels in the shoot and the root adjusts the carbohydrate

metabolism of the host to feed the AM-derived higher carbohydrate sink in the root system. This metabolic balance is obviously dependent on the fungal and plant partners as well as on the environmental conditions affecting the dynamic of the symbiosis.

Beyond the changed abundance and fluxes of nutrients, mycorrhizal associations involve changed levels of diverse plant hormones such as abscisic acid, auxins, cytokinins and jasmonate in the host plants (Drüge and Schönbeck, 1993; Ludwig-Müller, 2000; Hause et al., 2007). Even there is no plant hormone data available for mycorrhizal stock plants, recent studies on other plants support the view of **Druege et al. (2006)** that infection of stock plant roots with AMF can modify plant hormones in the shoot in a direction that may contribute to the observed enhanced survival and AR formation of cuttings. Leaves of tomato plants colonized by the AMF *Glomus mosseae* revealed lower abscisic acid (ABA) levels than those of control plants and this was assumed to contribute to the observed lower susceptibility of AM-plants to infection with *Botrytis cinerea* (Fiorilli et al., 2011). Interestingly, in the study of **Druege et al. (2006)** secondary infection of senescing leaves of *E. pulcherrima* with *B. cinerea* contributing to the die off of cuttings was lower in the cuttings harvested from the mycorrhizal plants. However, no differences in ABA levels were found in leaves and shoots of melon plants colonized with *Glomus intraradices* or *Glomus mosseae* (Martinez-Medina et al., 2010, 2011). Enhanced cytokinin levels in the leaves or shoot of mycorrhizal plants is a hormonal response, which has been repeatedly reported for different plant-fungus associations, even though these responses also depended on the stage of symbiosis (Allen et al., 1980; Drüge and Schönbeck, 1993; Goicoechea et al., 1997; Martinez-Medina et al., 2011). Interestingly, also the level of IAA was increased in the shoot of melon plants by root colonization with *Glomus intraradices,* a species which was also used in the stock plants of *E. pulcherima* (**Druege et al., 2006**), but decreased by root colonization with *Glomus mosseae* (Martinez-Medina et al., 2011).

Considering the roles of ABA and cytokinins as potential trigger and inhibitor of leaf senescence, respectively, their contrary roles in the regulation of stomatal conductance (Davies, 2010; Gan, 2010), and auxin as inductor of AR formation, mycorrhiza-induced changes in levels of these hormones are candidate factors, which may contribute to improved survival and AR formation of cuttings excised from AM plants. In general, the physiological consequences of the changed carbohydrate and/or hormonal condition of the shoot (cuttings) in response to the root symbiosis should depend on how long the response will be maintained after the initial inducer has been removed with the excision of cuttings from the mycorrhizal plant.

4.1.2 *P. indica* as stimulator of adventitious root formation

After our results had pointed to hormonal or other signal effects of *P. indica* on plants **(Druege et al., 2007**), there came up increasing support in literature that *P. indica* can produce plant hormones and modifies plant hormone related pathways in colonized roots, while nearly the whole orchestra of plant hormones seem to be involved (Franken, 2012). Interestingly, *P. indica* was shown to produce IAA and other indole-derivates in liquid culture (Sirrenberg et al., 2007; Vadassery et al., 2008; Hilbert et al., 2012). However, the effect of *P. indica* on auxin signal transduction and the involvement of auxin in *P. indica*-mediated growth obviously depend on the plant. In *Arabidopsis thaliana* seedlings, colonization of roots with the endophyte promoted root growth without changing auxin levels or the expression of auxin-regulated genes (Vadassery et

al., 2008; Lee et al., 2011). In contrast, inoculation of barley with *P. indica* enhanced the transcript levels of genes controlling auxin biosynthesis and repressed the expression of an auxin-repressed protein in roots (Schäfer et al., 2009). In another study with barley, however, strong reduction of the conversion of tryptophan to auxin in *P. indica* via silencing of the *piTam1* gene reduced the colonization of roots with the fungus, but the growth promotion was maintained (Hilbert et al., 2012). Roots of a subspecies of *Brassica campestris* responded to colonization with enhanced auxin levels and up-regulation of genes controlling auxin transport and components of the auxin response machinery (Lee et al., 2011). The important function of the up-regulated gene *BcAUX1,* which encodes a putative auxin influx carrier, was supported by the finding that overexpression of *BcAUX1* in *Arabidopsis* mimicked the promotive effect of *P. indica* on root growth. Interestingly, root growth in *B. campestris* was not stimulated by auxin itself but when an HPLC-enriched fraction of the fungal cell wall was applied (Lee et al., 2011).

Ethylene action in plants was also shown to respond to *P. indica* and to have important functions in the interaction between the endophyte and the host plant. In barley, two genes encoding ACO, which controls the final step of ethylene biosynthesis, were induced after inoculation, whereas three genes encoding components of the ethylene signalling cascade were down-regulated (Schäfer et al., 2009). Mutants of *Arabidopsis* defective in expression of either the ethylene receptor ETR1 or one down-stream component of the ethylene signal transduction pathway showed enhanced root colonization with *P. indica*, whereas the endophyte-mediated growth promotion was reduced or even turned into depression compared to wild type plants (Camehl et al., 2010). These results and the finding that overexpression of the ethylene response factor ERF1 strongly reduced root colonization and abolished benefits to the plants suggest that ethylene signalling controls the balance of beneficial versus non-beneficial traits in the symbiosis (Camehl et al., 2010). *P. indica* was further shown to produce relatively high amounts of cytokinins, levels of which were higher in roots of *Arabidopsis* when colonized with the endophyte (Vadassery et al., 2008). Furthermore, the growth promotion was dependent on trans-Zeatin biosynthesis and on the expression of the two cytokinin receptor genes *CRE1* and *AHK2*.

Taken together, these studies further support the hypothesis that *P. indica* promotes AR formation via changing plant hormone homeostasis and signalling in the cutting, with the auxin pathway being the first candidate. In this context, the question has to be answered, whether the endophyte promotes already the induction and initiation of roots or whether it accelerates root differentiation and growth. Time course analysis of the rooting zone of cuttings at levels of root anatomy, root colonization, plant hormone concentrations and expression of candidate genes have to be combined with chemical and/or genetic manipulations of plant hormone pathways at the fungus and/or plant side to unravel these interrelationships.

4.2 Nitrogen and carbohydrate interactions on rooting of cuttings

Concept 2: Nitrogen content in cuttings determines the availability of amino acids in the rooting zone utilized during AR formation.

The consisting findings on the limitation of AR formation by low nitrogen content in cuttings of three plant species has been confirmed also for *P. hybrida* (Santos et al., 2011; Zerche et al., 2016). The higher N_t levels measured in whole cuttings at time of harvest with increasing N

supply (**Druege et al., 2000, 2004**; **Zerche and Druege, 2009**) reflect a higher overall N abundance in the cuttings. Considering the demand of N for synthesis of diverse compounds particularly of proteins during AR formation, it can be expected that the positive influence of higher N_t in cuttings at time of excision on subsequent AR formation is at least partially mediated by higher provision of N compounds to the rooting zone. Higher N supply generally correlates with higher leaf amino acid levels in plants, while particular amino acids such as glutamine and also glutamate show specific responses, whereas nitrogen deficiency causes opposite reactions (Foyer et al., 2003; Scheible et al., 2004; Ruan et al., 2010). Leaves constitute important N source organs, from which N is remobilized via the phloem towards new developing sinks, while specific amino acids, in particular glutamine and asparagine, are predominant in phloem sieve tubes (Masclaux-Daubresse et al., 2010; Kant et al., 2011). Metabolic studies of AR formation in cuttings of *P. hybrida*, which had been produced under high nitrogen supply, revealed a transient depletion of amino acids, particularly of glutamine and asparagine in the stem base (rooting zone) during the induction phase, which was followed by a rise of amino acid levels, particularly of glutamate and aspartate, and also of protein levels during subsequent formation of ARs (**Ahkami et al.**, 2009, **2013**). This strongly suggests that synthesis of proteins and other N compounds in the rooting zone is subject to the influx of amino acids from the upper shoot, where the amount of proteins and amino acids provides the N source. Recent results in our group support the hypothesis that higher nitrogen content in the cutting contributes to an enhanced nitrogen remobilization and has a resource function for the development of new roots. Thus, enhanced N_t in petunia cuttings increased the allocation of N into mobile-pools, provided higher levels of amino acids, particularly of glutamine and glutamate in leaves and in the stem base at cutting harvest, and promoted the differentiation of new meristematic cells into fully developed ARs (Zerche et al., 2016). In this context, it has been shown in leaves of *Lolium perenne*, that nitrogen deficiency prolonged cell cycle duration and delayed post-mitotic cell elongation (Kavanova et al., 2008).

Concept 3: Higher nitrogen nutrition may promote AR formation via enhanced leaf carbohydrate export and plant hormone related pathways.

For intact plants of several species, a clear positive relationship has been documented between leaf N concentration and the light saturated rate of photosynthesis (Pons and Westbeek, 2004; Uribelarrea et al., 2009; Peng et al., 2014). Considering the importance of current photosynthesis for AR formation in cuttings **(Rapaka et al., 2005**; **Druege and Kadner, 2008**; **Klopotek et al., 2012**), it is conceivable that the positive effect of initial N content on subsequent AR formation may be in part mediated by enhanced photosynthesis of nitrogen-rich cuttings providing higher carbohydrate source capacity in the cutting to meet the carbohydrate demand in the rooting zone. However, the relationship between N and carbohydrate metabolism is much more complex, while the system is fine-tuned according to the inputs of C and N via distinct N- and C-sensing systems (Foyer et al., 2003). Uptake of nitrogen has a strong influence on shoot-root carbon allocation in plants, involving intriguing interactions among nitrate, sugars and many other long-distance signals such as cytokinins and auxin (Ruan et al., 2010; Wang and Ruan, 2016). The finding that enhanced AR formation of nitrogen-rich cuttings was correlated with a higher sucrose/starch ratio at harvest might indicate that the higher N supply and assimilation stimulated AR formation also via an enhanced carbohydrate export from leaves which may promote translocation of carbohydrates and co-transported amino acids to the rooting zone

(**Druege et al., 2000**). However, up-to-date no data is available on the influence of nitrogen supply to stock plants or nitrogen content on processes of carbohydrate allocation in cuttings. In plants of *Lolium perenne,* Lattanzi et al. (2012) analysed the allocation of assimilated ^{13}C to different carbohydrate fractions in source leaves under the influence of continuous light and contrasting nitrogen supply. Modelling the proportions of the leaf sucrose pool leaving to carbohydrate export, they calculated mean export percentages of 24% versus 15% for high and low nitrogen supply, respectively, but the difference was not statistically significant.

Regarding the important role of plant hormones in AR formation, **Druege et al. (2000**) suggested that the impact of high N supply and high N_t in cuttings on AR formation may be also mediated by plant hormones. Since the different N dosages were realized by repeated supply of different concentrations of ammonium nitrate in the irrigation water **(Druege et al., 2000, 2004**; **Zerche and Druege, 2009**), despite the expected nitrification in the substrate, hormonal effects of both nitrate and ammonium have to be considered.

Concerning auxin as important inductor of ARs, the biosynthesis of IAA depends on the amino acid L-tryptophan and thus on nitrogen (Ljung, 2013). Nitrogen supply further has a strong influence on auxin transport in plants. In lateral roots of *Arabidopsis*, the nitrate transporter NRT1.1 obviously favours basipetal (shoot-ward) transport of auxin from the root tip, while this function is suppressed by nitrate (Krouk et al., 2010). However, there are conflicting indications in literature how nitrogen fertilization may affect shoot-to-root transport of auxin. Increasing the concentration of nitrate in the nutrient solution of maize plants to 5 mM and higher reduced the concentration of IAA in phloem exudates of shoots compared with 0.05 and 0.5 mM nitrate (Tian et al., 2008). Accordingly, application of 1 mM nitrate to the roots of maize after one day reduced the transport of ^{3}[H] IAA from shoot to roots when compared to nitrate-free cultivation (Liu et al., 2010). Similarly to these results, *Arabidopsis thaliana* plants revealed a higher auxin transport in apical segments from inflorescence stems, when receiving 1.8 mM nitrate compared with 9 mM nitrate for four weeks (De Jong et al., 2014). By contrast, shoot-to-root auxin transport in seedlings of *Medicago truncatula* was enhanced with increasing shoot N concentration, after they had been cultivated under variable supply of nitrate and CO_2 for 10 days (Jin et al., 2012). Considering the conflicting results between different studies, Jin et al. (2012) suggested that the detailed relationship between nitrate treatment and auxin transport might depend on plant species, time of measurement, nitrate concentration, and method of nitrate application. Nitrogen nutrition influences also auxin-signalling in plants. Gifford et al. (2008) identified the auxin response factor ARF8 in root pericycle cells of *Arabidopsis*, which was induced by nitrate via release from the repression by a micro RNA. Also in the root, the auxin receptor gene *AFB3* was found to be nitrate-inducible (Vidal et al., 2010).

Studies on intact plants (Kiba et al., 2011; Wang and Ruan, 2016) suggest that the cytokinin homeostasis and signalling might be altered in nitrogen-rich compared to low-nitrogen cuttings. The finding that nitrogen supply enhanced cytokinin levels in roots, shoots, excised leaves and also in xylem exudates of diverse plant species indicate that cytokinins function as a root-to-shoot long-distance signal of nitrogen supplement (reviewed in Sakakibara et al., 2006; Kiba et al., 2011; Wang and Ruan, 2016). The positive effect of nitrogen supply on cytokinin level has already been highlighted at the level of cytokinin biosynthesis controlling genes (Kiba et al., 2011). Transcript accumulation of *AtIPT5,* which encodes a rate-limiting ADP/ATP isopententyl transferase, was in the pericycle and primordia of lateral roots correlated with the concentrations

of nitrate and ammonium in the growth medium (Takei et al., 2004). Another isogene of the same family, *AtIPT3, which is* particularly expressed in the phloem throughout the plant, was upregulated within 1h after application of nitrate to mineral-starved plants, while also detached shoots responded to this treatment (Miyawaki et al., 2004). Furthermore, studies on rice indicate that nitrogen supply mediates also the expression of genes controlling the cytokinin response in an organ-specific manner (Ding et al., 2014). Strigolactones have been shown to increase systemically in mono- and dicotyls in response to nitrogen deficiency (Steffens and Rasmussen, 2016 and references therein). Thus, enhanced AR formation with enhanced nitrogen supply to stock plants may also be the consequence of the release from the inhibitory influence of strigolactones.

In conclusion, there is a high chance that nitrogen-mediated changes in plant hormone homeostasis and/or signalling are involved in the enhanced AR formation in nitrogen-rich cuttings. However, the complexity of these relationships in intact plants and the strong dynamic of plant hormone concentrations and signalling induced by the wounding and isolation of cuttings from the whole plant (**Ahkami et al., 2013**; Da Costa et al., 2013; **Druege et al., 2014**) up to now do not allow to deduce a distinct hypothesis, how plant hormones may contribute to N-stimulated AR formation in cuttings.

Concept 4: High nitrogen supply decreases carbohydrate levels and provokes carbohydrate shortage in cuttings when photosynthesis is low.

The consistent finding with three plant species that enhanced N-uptake and N-content in the cuttings at harvest were negatively correlated with the level of carbohydrates, particularly of starch in cutting tissues (**Druege et al., 2000, 2004**; **Zerche and Druege, 2009**) stays in accordance with similar findings on plants of other plant species reflecting that carbohydrate biosynthesis and N-assimilation into amino acids are processes competing for inputs of reduced carbon and energy (Huber and Kaiser, 1996; Foyer et al., 2003; Cai et al., 2012). The finding in our studies (**Druege et al., 2000, 2004**; **Zerche and Druege, 2009**) that the lower starch levels in nitrogen-rich cuttings at time of harvest corresponded with a stronger sugar depletion during subsequent dark storage, suggest starch as important carbohydrate fraction to support sugar homeostasis in cuttings during post-harvest phases which do not allow for photosynthesis. As a result, higher nitrogen reduces the sugar abundance particularly in the leaves when the cuttings are exposed to post-harvest dark-storage (**Druege et al., 2000, 2004**; **Zerche and Druege, 2009**). The latter obviously impedes subsequent AR formation and interferes with the positive nitrogen effect when the physiological cutting condition (affected by the plant genotype and the light adaptation of the photosynthetic apparatus) or environmental conditions (e.g. low light intensity) impede substantial photosynthesis (see also next chapter).

4.3 Involvement of carbohydrate source and sink relations in rooting of cuttings

Concept 5: In dependence on initial carbohydrate reserves and current photosynthesis, the carbohydrate availability in source leaves determines sucrose export to the new sink in the stem base, where sucrose or other generated sugars control AR formation.

The positive correlations found between the levels of sugars, particularly of sucrose, in the leaves at time of planting and during the early rooting period, the intermediate levels of sugars in the stem base, and the final intensity AR formation under the influence of storage stress the importance of a high and steady export of carbohydrates from the source leaves as important requirement for a high intensity of AR formation in the stem base of leafy cuttings (**Druege et al., 2004**; **Rapaka et al., 2005**; **Druege and Kadner, 2008**; **Zerche and Druege, 2009**). Furthermore, the observed interdependency of carbohydrate status at time of planting and photosynthesis during rooting in the limitation of AR formation indicates that leaf carbohydrate export is a function of both initial reserves and current assimilation (**Rapaka et al., 2005**; **Druege and Kadner, 2008**). In this context, photosynthesis and net carbon assimilation are obviously influenced not only by the environmental factors particularly by light intensity, but also by the plant genotype (**Rapaka et al., 2005**; **Druege and Kadner, 2008**; **Klopotek et al., 2012, 2016**). Whereas pelargonium cuttings showed only a weak photosynthetic activity (**Druege and Kadner, 2008**), cuttings *of P. hybrida* cv. 'Mitchell' responded to similar environmental conditions with a much higher net photosynthesis (**Klopotek et al., 2012, 2016**). Correspondingly, AR formation in cuttings of petunia was not reduced by dark storage despite a strong depletion of carbohydrates but was strongly promoted being associated with a strong accumulation of carbohydrates in cutting tissues during the subsequent light period **(Klopotek et al., 2010**).

Confirming our results, new studies of other authors support the important contribution of sufficiently high light intensity on AR formation in leafy cuttings (Lopez and Runkle, 2008; Zobolo, 2010; Park et al., 2011; Currey et al., 2012; Hutchinson et al., 2012). Research on cuttings of four plant species provided coherent pictures on the positive relationships between light intensity or DLI, the carbohydrate levels in the stem base during rooting and the final intensity of AR formation (Currey and Lopez, 2015; Tombesi et al., 2015). Considering that earlier investigations of photosynthesis in cuttings were mostly done on single leaves or single cuttings outside of the growth environment and revealed initially low photosynthetic activity before first roots emerged from the stem base, **Klopotek et al. (2012)** already stressed the risk of water deficit stress which may influence the light response of photosynthesis. Tombesi et al. (2015) measured side effects of different light intensities in the greenhouse on air temperature, air vapor pressure deficit, stomatal conductance and water potential of cuttings during rooting. With regard to the data and earlier conflicting results on the light response of cuttings, they pointed out the importance of maintaining optimum vapor pressure deficit by adjusting the light intensity and the water supply of cuttings during rooting.

The obvious dependency of AR formation in cuttings on leaf sugars, particularly leaf sucrose (**Druege et al., 2000**; **Rapaka et al., 2005**; **Druege and Kadner, 2008**) and the strong indication that sucrose is cleaved to the monosaccharides glucose and fructose in the rooting zone starting soon after excision of cuttings (**Ahkami et al.,** 2009, **2013,** 2014) highlight the important function of sucrose as "assimilate vehicle" between the carbohydrate source in the fully developed leaves and the newly established sink in the rooting zone. Further, there is indication from cuttings of *Pinus radiata* and from *P. hybrida* that starch granules which intermediately accumulate in close proximity to the sites of new primordia formation serve as transient depots of the delivered sucrose to provide for the enhanced carbohydrate demand when the ARs are formed (Li and Leung, 2000; Ahkami et al., 2009). Kircher and Schopfer (2012) recently highlighted the important function of photosynthetic sucrose in root growth during early seedling development in

Arabidopsis. By combining targeted light application with inhibition of photosynthetic assimilation, use of mutants in photomorphogenesis and phloem-loading of sucrose and sucrose feeding, they showed that cotyledon-derived photosynthetic sucrose controls growth of the primary root. Further considering postulated signal functions of sucrose in lateral root development (MacGregor et al., 2008) and in P-deficiency-stimulated promotion of root development (Hammond and White, 2011), the authors suggested that sucrose might function not only as fuel but also as inter-organ signal to initiate root growth during early seed development which was obviously independent of shoot-derived auxin (Kircher and Schopfer, 2012) (see also chapter 4.4).

The high expression and activities of invertases found already during the first hours post excision and the rises in glucose and fructose levels in the stem base of cuttings following a transient depletion indicate that the arriving sucrose is immediately utilized during the induction phase already (**Ahkami et al.,** 2009, **2013**; **Klopotek et al., 2010, 2016**). That diverse sugars can promote AR formation is supported by several *in vitro* studies where sugar applications promoted AR formation in cuttings (Druege, 2009 and references therein; Yin et al., 2013; Abul-Soad and Jatoi, 2014). However, the *in vitro* studies did not provide a coherent picture on particular contributions of specific sugars to AR formation. Takahashi et al. (2003) reported similar promotive effects of glucose, fructose and sucrose on AR formation in hypocotyls of *Arabidopsis*. Correa et al. (2005) studied the influence of individual sugars on AR formation in two *Eucalyptus* species when added during different phases of AR formation. They found a promotive effect of glucose when added during the induction phase, whereas sucrose promoted AR formation when added during the root formation phase. Similarly, in *Bambusa nutans* the number of formed AR initials was enhanced when glucose was added to the auxin-containing induction medium (Yasodha et al., 2008). However, among four different sugars glucose was ineffective in AR induction in *Physocarpus opulifolius*, whereas fructose had the most stimulating influence (Ilczuk et al., 2013).

Carbohydrates, especially sugars can promote AR formation by diverse functions (Druege, 2009). They represent important and highly mobile providers of both the energy and C used to synthesize all other organic compounds essential for AR formation. According to this function, activities and gene expression of important enzymes of the glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, the amino acid metabolism, and the nucleotide biosynthesis show a strong induction during AR formation in petunia particularly during the late induction phase and the root formation phase (**Ahkami et al.,** 2009, **2013**, 2014). Sugars are further essential components of the osmotic potential in plant cells, which regulates cell turgor and stability and drives the growth-limiting fluxes of water and solutes. However, far beyond these chemical and physical functions sugars act as signals and mediate the development, growth and stress responses of plants by modulating gene expression (Rolland et al., 2006; Lastdrager et al., 2014). There is increasing evidence that sugars can regulate specific developmental programmes and transitions via genes that control meristem maintenance and identity (Eveland and Jackson, 2012). For example, recent work has shown that exogenous sucrose can compensate for regulators of meristem maintenance in the shoot and the root (reviwed in Eveland and Jackson, 2012). Considering also the linkage between sugar and plant hormone signalling in plant development (Ljung et al., 2015), the putatively involved signalling pathways will be discussed in chapter 4.4.

Concept 6: Advanced establishment of the carbohydrate sink in the rooting zone and mobilization of nitrogen resources in the cutting support meristemoid formation under darkness and contribute to dark-stimulation of AR formation.

Expression and high activities of invertases particularly of INVcw are obviously involved in the establishment of the new sink in the stem base of cuttings (Ahkami et al., 2009, 2014). The study of **Klopotek et al. (2016**) contrasted the expression and activities of invertases in the stem base of petunia cuttings with those in the shoot apex as competing utilization sink under the influence of dark exposure. The data provide evidence that dark exposure of cuttings specifically stimulates the expression of *PhINVcw2* in the stem base coinciding with a rise of INVcw activity in the same tissue whereas transcript and enzymatic activity remain at low levels in the shoot apex. Considering further the shift in expression of three isogenes of INVvac and INVcyt towards the stem base under darkness compared to light, the results strongly support the model, that these changes drive an enhanced sink strength of the rooting zone compared to the shoot apex, which contributes to the increased carbon allocation towards the developing roots when the cuttings are exposed to light after the dark treatment (Figure 2, **Klopotek et al., 2016**). Other studies on diverse plants confirm light-, temperature- and tissue-specific expression of invertases, which are further dependent on plant hormones (Yun et al., 2002; Liao et al., 2013; Gao et al., 2014; Rabot et al., 2014). **Agulló-Antón et al. (2011**) found that auxin application to carnation cuttings before planting stimulated the subsequent accumulation of sugars in the stem base of non-stored cuttings but had a less pronounced effect on previously cold dark stored cuttings. This suggests that auxin action is involved in the enhanced sink strength and invertase activity in the stem base of dark treated cuttings (see also chapter 4.4).

A recent study of Zerche et al. (2016) showed that dark exposure also enhances the soluble amino-N and amide-N pools in petunia cuttings at the expense of insoluble protein-N. Furthermore, dark exposure strongly raised the levels of amino acids in source leaves and the stem base, which was also dependent on the total nitrogen content of cuttings provided by the fertilization of stock plants. In particular, levels of aspartate, arginine, and asparagine increased during dark exposure, while the latter two amino acids showed the most pronounced increase in source leaves where also glutamate showed a transient accumulation. The data as whole supports the view that dark-induced carbohydrate depletion induces proteolysis particularly in source leaves leading to accumulation and mobilization of amino acids in the cutting. While free asparagine and arginine – having high N to C ratios – function as N storage compounds buffering the high ammonium release from proteolysis, the phloem-mobile amino acid asparagine (Masclaux-Daubresse et al., 2010; Kant et al., 2011), which showed a specific profile during the course of AR formation **(Ahkami et al.,** 2009, **2013**), was suggested as candidate transport unit of proteolysis-derived organic N (Zerche et al., 2016). In the stem base, amino acids and released compounds may contribute to the dark-induced stimulation of AR formation via resource and possibly signal functions for example involving arginine-derived NO signalling (Zerche et al., 2016, see also chapter 4.4.).

In conclusion, dark exposure enhances 1) the sink competiveness of the rooting zone and 2) the nitrogen source in the cutting by raising the level of mobile N (**Klopotek et al., 2016**; Zerche et al., 2016). Both processes can be expected to support the observed formation of meristemoids under darkness and contribute to the accelerated development of ARs under subsequent light

(Klopotek et al., 2010, 2016). However, the positive effects of dark exposure on AR formation were found under conditions that allowed sufficient photosynthesis after the dark period to power fast recovery from dark-induced carbohydrate depletion (**Agulló-Antón et al., 2011**; **Klopotek et al., 2010, 2012, 2016**). In contrast, weakly photosynthesizing cuttings of pelargonium under low light and high temperature showed prolonged carbohydrate depletion after dark exposure which obviously then acted as primarily bottleneck limiting AR formation (**Druege et al., 2004**; **Rapaka et al., 2005**; **Druege and Kadner, 2008**).

4.4 Involvement plant hormone pathways in leaf senescence and adventitious root formation in cuttings

Our data indicated that ethylene biosynthesis and signalling has an ambivalent role in cuttings. It contributed to leaf senescence in the sensitive plant species pelargonium **(Kadner and Druege, 2004**) as found by other authors (Serek et al., 1998; Mutui et al., 2005) but also turned out as important regulative factor for the AR formation in the stem base in pelargonium and petunia (**Kadner and Druege, 2004**; **Druege et al., 2014**).

Concept 7: Wound and stress-induced ethylene and JA biosynthesis and signalling may trigger leaf senescence in sensitive species and stimulate AR formation in cuttings.

Wounding at the cutting site is an inevitable factor involved in the physiology of cuttings and wound stress is one of the strongest factors triggering ethylene biosynthesis particularly in vegetative tissues at the levels of ACC biosynthesis and oxidation (Druege, 2006). In accordance to our finding on the contribution of ethylene perception to leaf senescence in pelargonium cuttings (**Kadner and Druege, 2004**), the important function of ethylene in senescence of cutting leaves in terms of leaf yellowing or leaf abscission has been confirmed for large array of plant species including *Codiaeum variegatum*, *Lantana camara*, *Begonia hybrida*, and *Portulaca oleracea* (Müller et al., 1998; Rapaka et al., 2007a, 2007b; Leatherwood et al., 2016). Investigations of *Arabidopsis* mutants defective at different levels of ethylene perception and signalling have highlighted the stimulatory role of ethylene in leaf senescence particularly in darkness (Kim et al., 2015). Similar to our study (**Kadner and Druege, 2004**), postharvest blocking of ethylene receptors in cuttings of *P. hortorum* by application of 1-MCP substantially increased ethylene production in vegetative cuttings of the same species and of *Pelargonium peltatum*, *Euphorbia pulcherrima, Lantana camara, Impatiens hawkeri* and *P. hybrid*a (Rapaka et al., 2007b, 2008; Leatherwood et al., 2016). Our explanation that this is probably attributed to the interruption of auto-inhibition of ethylene biosynthesis was supported by Mutui et al. (2007) who found that ethylene production in pelargonium cuttings was reduced by application of ethylene. However, when analysing the expression of four genes encoding ACS and two genes encoding the negative acting ethylene receptors ETR1 and ETR2 by semi-quantitative RT-PCR, the authors did not find indication that transcriptional regulation of ACS or the ethylene receptors was causally involved in the ethylene response. This may indicate that the auto-inhibitory loop of ethylene biosynthesis in pelargonium cuttings is controlled by other members of the gene families, at other levels of the biosynthetic or signalling pathways or even post-transcriptionally. Nevertheless, up-regulation of one *ACS* gene by ABA application and increased ethylene production and expression of the *PhETR1* gene after application of thidiazuron, a substance with

high cytokinin activity, confirm earlier found promotive influences of ABA and inhibitory influences of thidiazuron on dark storage induced leaf senescence in the same species (Mutui et al., 2005).

Overall, the data indicates that leaf senescence in cuttings is subject to crosstalk between different hormones while ABA and cytokinins have agonistic and antagonistic functions on the ethylene pathway, respectively. Furthermore, considering the increasing support in literature for a positive role of jasmonic acid (JA) in leaf senescence on the one hand (Kim et al., 2015) and the strong and systemic accumulation of JA in cuttings shortly after excision (Ahkami et al., 2009) on the other hand, also stimulation of this pathway may contribute to leaf senescence in cuttings.

Confirming our results on the response of AR formation in *Pelargonium hortorum* to ethylene application and blocking of ethylene perception by MCP (**Kadner and Druege, 2004**), applications of aminoethoxyvinylglycine (AVG) and STS, inhibitors of ACS activity and ethylene perception, to de-rooted seedlings of petunia reduced the number of ARs, whereas application of the ethylene precursor ACC had the opposite effect (**Druege et al., 2014**). The results demonstrate that ethylene biosynthesis and signalling have positive functions in AR formation in both species. In addition to wounding, the isolation of cuttings from the whole plant includes cutting off from the water flow and from all other root-sourced influxes of signals and resources. Furthermore, there exists extensive crosstalk between auxin and ethylene at the levels of metabolism, transport and signalling (Negi et al., 2010; Muday et al., 2012; Pacheco-Villalobos et al., 2013). Thus, we consider the wounding and deficiencies in water and minerals as important environmental factors and the IAA accumulation during the induction phase (**Ahkami et al., 2013**) as hormonal factor for the observed stimulation of the ethylene biosynthetic pathway in the stem base (**Druege et al., 2014**). The positive function of ethylene in AR formation of cuttings is further supported by recent other studies on diverse plant species. Leatherwood et al. (2016) confirmed the reduced AR formation in *Pelargonium hortorum* in response to MCP and further showed that blocking of ethylene perception in six other plant species delayed AR formation in term of percentage of rooted cuttings. Liu et al. (2013) monitored a strong increase of ethylene evolution from the stem base of chrysanthemum cuttings peaking at 1 dpe. Furthermore, application of $AgNO_3$ as inhibitor of ethylene perception before planting the cuttings inhibited AR formation. Compared to a weak rooting cultivar, a good rooting cultivar of carnation accumulated higher levels of ACC at 24 and 54 hpin, and ACC levels could be enhanced by applying auxin to stimulate AR formation (Villacorta-Martin et al., 2015).

In petunia, many ERFs were continuously up-regulated during AR formation and also induced in wounded leaves (**Druege et al., 2014**). ERFs regulate ethylene-responsive genes and are considered to affect developmental processes, while their expression responds to hormones including ethylene and to many different external stimuli as e.g. abiotic stress factors (Ohme-Takagi et al., 2000; Licausi et al., 2013). However, considering the plenty of regulated ERFs and also ethylene biosynthetic genes without marking specific time points, it appears that unlike the obvious master control by auxin, ethylene seems to be important to stimulate AR development in cuttings but not to control the process per se (**Druege et al., 2014**). According to this view, in cuttings of *Catalpa bungei* which received a pulse treatment with auxin after excision, two genes encoding ACO and three of four genes encoding ERFs were continuously up-regulated in the stem base between 1 dpe and 35 dpe marking the induction and elongation of ARs, respectively (Wang et al., 2016).

The lacking effect of ethephone, an ethylene releasing substance, on AR formation and the observed inhibitory effect of both ACC and ethephone on average root length in petunia indicates a narrow range of optimum ethylene levels for induction of ARs and that high ethylene levels reduce AR elongation (**Druege et al., 2014**). This is in agreement with findings on ARs and lateral roots in other plant species (Riov and Yang, 1989; Negi et al., 2008). According to this concept, application of 0.5 µl L^{-1} of ethylene for 4 dpe to cuttings of *P. hortorum* enhanced the percentage of rooted cuttings and the dry mass of formed ARs in two cultivars, whereas 1 µl L^{-1} of ethylene reduced the number and fresh mass of formed ARs in one of the cultivars (Mutui et al., 2010). The effect of high ethylene concentrations may be indirect via stimulating biosynthesis and transport of auxin into the elongation zone, where it inhibits root elongation (Muday et al., 2012). In conclusion, ethylene biosynthesis and signalling seems to be an important factor contributing to the formation of ARs in cuttings (Figure 6, Druege et al., 2016).

According to the general wound response of the JA biosynthetic pathway, Ahkami et al. (2009) found a strong accumulation of JA in the stem base of petunia during the induction phase, peaking at 0.5 hpe already, while the transient accumulation preceded the RNA accumulation of a member of the cell wall invertase gene family and the rise in the corresponding enzymatic activity. Jasmonates may stimulate cell wall invertases as shown in petals of rose (Horibe et al., 2013). Lischewski et al. (2015) recently tested the function of JA in AR formation in petunia cuttings. A strong reduction of transcripts and activity of petunia allene oxide synthase, the rate limiting enzyme in JA biosynthesis, significantly reduced the levels of JA and its bioactive conjugate (+)-7-iso-jasmonyl isoleucine in the cuttings. This reduced the numbers of root primordia formed at 7 dpe and the number of ARs determined at 21 dpe confirming the positive role of JA in AR formation of petunia. Further analysis of hormone levels, cell wall invertase activity and related transcripts at time points of expected maxima did not indicate that JA functioning during AR formation is mediated via auxin homeostasis, ethylene biosynthesis or carbohydrate metabolism. The authors concluded that JA might act as an accelerator of AR formation. According to these results, Rasmussen et al. (2015) recently showed that AR formation in rooting-competent vegetative cuttings of pea exhibited an early rise in JA during the induction phase. This rise was delayed in low-rooting cuttings, which apical meristem had switched to floral identity. Interestingly AR formation in the rooting-competent cuttings could be enhanced by a pulse treatment with JA during the first 6 hpe. Considering also the positive effects of JA observed on AR formation in potato cuttings (Ravnikar et al., 1992), it appears that JA has a stimulating function in excision-induced AR formation particularly during the induction phase (Figure 6, Druege et al., 2016). This is in contrast to the negative role of JA in de-etiolation-induced AR formation in intact hypocotyls of *Arabidopsis* (Gutierrez et al., 2012). The particular roles of JA during the other phases of AR are, however, still in question (Figure 6). Even though the auxin maximum in the stem base of petunia was not altered by inhibited JA biosynthesis (Lischewski et al., 2015), interrelationships between JA and auxin homeostasis and signalling, rarely understood yet (Perez and Goossens, 2013), should be further considered in future studies.

Concept 8: PAT-dependent early influx of IAA and fine tuning of auxin biosynthesis, transport, metabolism and conjugation regulate auxin homeostasis in the stem base to promote AR induction and formation.

Considering the PAT-dependent early accumulation of IAA in the stem base of petunia cuttings peaking at 24 hpe (**Ahkami et al., 2013**) and the transcriptional regulation of auxin carrier genes, **Druege et al. (2014)** suggested a particular role of specific efflux carriers during AR induction. They further postulated that auxin accumulation at least partially reflects a non-transcriptionally regulated overflow of auxin resulting from the interruption of the basipetal auxin drain after excision of the cuttings (Figure 6, **Druege et al., 2014**; Druege et al., 2016). Also Agulló-Antón et al. (2014) suggested that a similar IAA peak observed in stem base of carnation cuttings is the consequence of the breakdown of the vascular continuum. Considering that auxin transport data from *Arabidopsis* and pea indicate considerable excess capacity in stems to transport widely varying amounts of auxin (Renton et al., 2012), the already active transport machinery should be sufficient to generate the auxin peak.

The important role of PAT and early auxin accumulation in AR formation in cuttings (**Ahkami et al., 2013**) is supported by several recent other studies. Sukumar et al. (2013) showed that localized induction of the *ABCB19* auxin transporter contributes to excision-induced AR formation in *Arabidopsis* hypocotyls while mutations of *ABCB19*, *PIN1*, *PIN3-3* and *PIN7* genes caused significant reductions in AR formation. High rooting capacity of vegetative pea cuttings was associated with an early peak of IAA in the stem base, which was absent in low rooting cuttings with a floral apical shoot meristem (Rasmussen et al., 2015). In de-rooted seedlings of mung bean, a *PIN1* homologue was up-regulated during the induction and initiation (24 hpe) phase whereas a putative auxin influx transporter gene (*LAX4*) showed opposite responses (Li et al., 2015a). When cuttings of carnation were immediately dark stored at 2°C–5 °C after excision, IAA in the stem base peaked at 8 hpe (Agulló-Antón et al., 2014). When those cuttings were subsequently exposed to higher temperature and diurnal light, two putative influx and efflux transporter genes, *DcLAX2* and *DcPIN3*, were up-regulated after 15 hours (23 hpe), while the expression of both genes was further enhanced when auxin was applied during the 15 hour period (Agulló-Antón et al., 2014). Under same experimental conditions, Villacorta-Martin et al. (2015) combined hormone analysis with RNA-seq-based monitoring of the transcriptome in the stem base of cuttings of two carnation cultivars with contrasting efficiencies of AR formation. At 0 hpin (23 hpe), a high IAA level was recorded only in the stem base of the good rooting cultivar, which, however, was strongly reduced by auxin application. The auxin treatment accelerated the activation of cell division and caused a higher number of root initials within the cambium of both cultivars. Because the poor rooting cultivar exhibited higher levels of trans-zeatin between 0 until 54 hpin and considering the inhibitory role of cytokinins on AR induction, Villacorta-Martin et al. (2015) concluded that the weak rooting was related to the low ratio of auxin vs. cytokinin levels.

There is also indication, that physiologically based supra-optimal IAA levels during the induction phase may inhibit AR formation in cuttings. Thus, a lower rooting capacity and enhanced callus formation of cuttings of *Prunus subhirtella* excised from the top of a tree compared to bottom cuttings was associated with higher IAA levels at the severance day, while the levels of indole-3-acetyl aspartate, indole-3-methanol and 2-oxindole-3-actic acid indicate a preferential conversion of IAA through the oxidation pathway in the bottom cuttings (Osterc et al., 2016). Interestingly, a

very recent study of Ribeiro et al. (2016), who combined QTL analysis of a segregating population of *Populus* with genome and transcriptome data, found further indication that not only the dynamic of auxin biosynthesis and metabolism but further the involved auxin biosynthetic pathways determine the intensity of AR formation in cuttings. When compared to the QTL category of intensively rooting cuttings, individuals in the QTL category of poor rooting cuttings revealed during later stages of AR formation higher expressions of the two genes TRYPTPHAN SYNTHASE ALPHA CHAIN and SUPERROOT2, which putatively control the biosynthesis of tryptophan as IAA precursor and the direction of the tryptophan pathway flux to the synthesis of indole glucosinolates, respectively (Ribeiro et al., 2016). In petunia, the prominent down-regulation of genes encoding auxin efflux transporters after the peaking of IAA and the corresponding pronounced up-regulation of most genes encoding GH3 proteins and peroxidases suggest that the early excision-induced accumulation of IAA triggers a transcriptional change favouring a subsequent decrease in IAA level which is required for root differentiation after the induction phase (**Ahkami et al., 2013**; **Druege et al., 2014**). According to this concept of auxin-induced down-regulation of auxin levels, in the stem base of cuttings of *Catalpa bungei* two genes encoding PIN3 and PIN8 and two genes coding for GH3.1 and GH3.6 were continuously down-and upregulated, respectively, after auxin application (Wang et al., 2016).

Considering the strong feedback between auxin level and regulation of auxin carriers (Habets and Offringa, 2014), **Druege et al. (2014)** hypothesized that the differential expression of auxin carriers and their controlling kinases after excision of cuttings is partially auxin-induced as the result of the initial auxin overflow. Bennett et al. (2014) considered self-organizing patterns of auxin transport as important factors guiding plant development, e.g. vascular patterning and initiation of leaf primordia in angiosperm shoot meristems. They distinguished between i) 'canalization', in which an initially broad domain of auxin transporting cells is reduced to a narrow 'canal' because a subset of cells become progressively more polarized and competent to transport auxin, and ii) 'maximization', in which auxin is transported towards cells containing higher auxin levels leading to formation of an auxin 'maximum'.

Considering these relationships, Druege et al. (2016) recently postulated that the initial auxin peak in the stem base of cuttings initiates self-regulatory auxin canalization towards and maximization in particular target cells, there starting the program of AR formation (Figure 6).

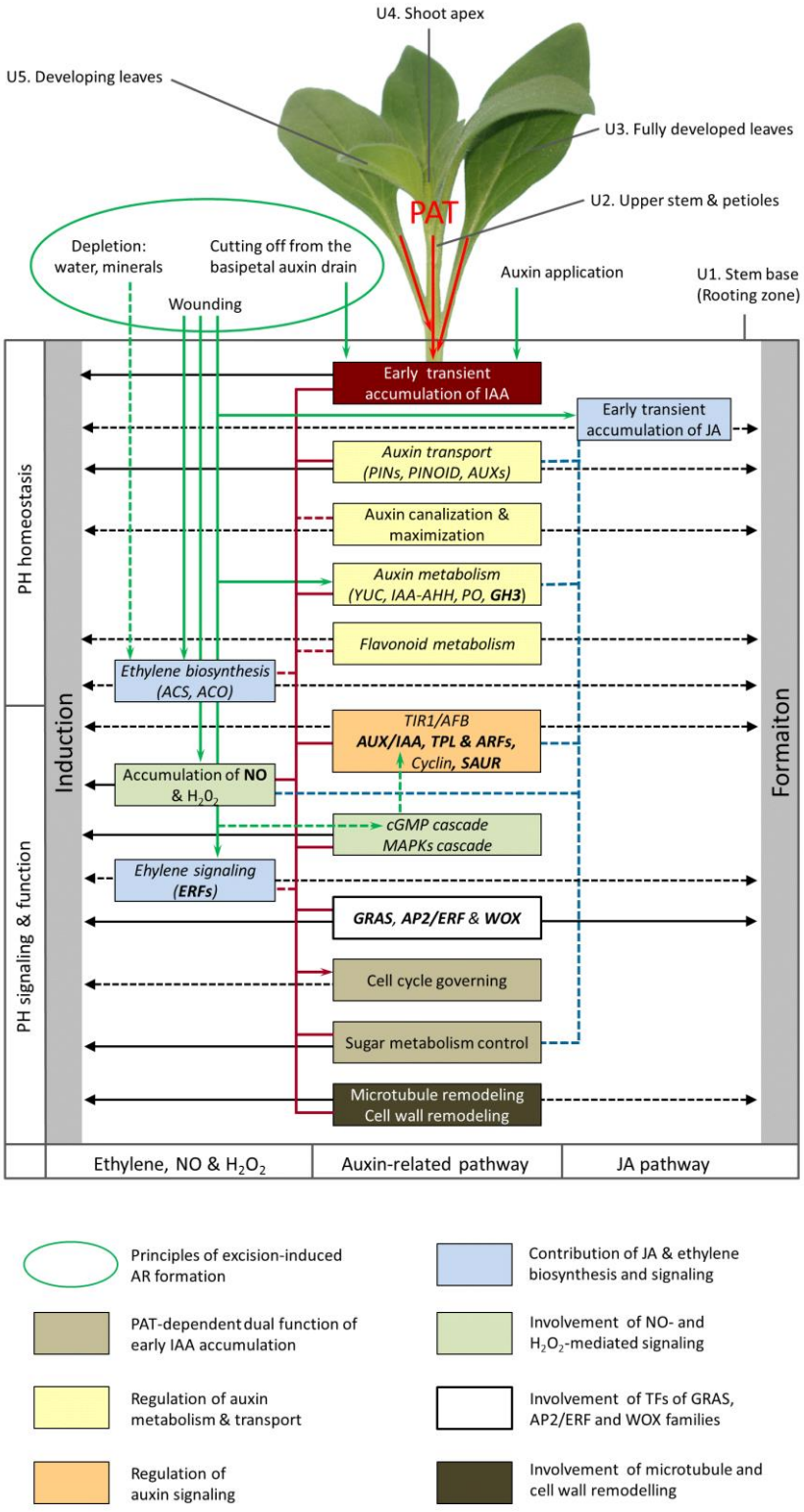

Figure 6 General model of important physiological units of shoot tip cuttings and regulative factors controlling ethylene, auxin and JA homeostasis, signalling and function in AR formation. Components with postulated phase-specific regulative character and crossroad functions between different plant hormones (PH) are indicated by italic and bold letters, respectively. Black arrows indicate evident or hypothetic (dashed lines) functions in induction and formation of ARs. Green arrows indicate evident (supported

by data on cuttings) and hypothetic (supported by other data, dashed lines) factors stimulating accumulation of IAA (PAT-dependent), JA and NO, auxin biosynthesis and mobilization, and ethylene biosynthesis and signalling. Red lines indicate evident and hypothetic (dashed lines) linkages between components of ethylene and auxin biosynthesis, signalling and function. Blue dashed lines indicate linkages between JA and auxin homeostasis and signalling and invertase activation. Function of units (not complete): U1: rooting zone, U2: transport route of hormones and others, U3: carbohydrate source, potential source of auxin, U4: carbohydrate sink competing to the rooting zone, potential source of auxin, U5: carbohydrate sink, potential source of auxin. Modified after Druege et al. (2016).

The important role of auxin allocation to particular cells as principle of AR induction is supported by studies of Sukumar et al. (2013) on de-rooted *Arabidopsis* seedlings of a *pGH3-2:GUS* reporter line, where auxin maxima were detected in pericycle cells, the sites for subsequent root primordia formation. Furthermore, recent studies by Della Rovere et al. (2013) on isolated Thin Cell Layers (TCLs) and intact hypocotyls of *Arabidopsis* highlighted the tissue-specific auxin control of cell fate and differentiation during AR formation. In primary and lateral roots, a group of less mitotically active cells, the Quiescent Center (QC) is essential for maintaining undifferentiated cells in the apical meristem (Petricka et al., 2012). Localization of expression of a QC marker gene and of genes for candidate TFs and auxin transporters by using promotor-reporter constructs together with immuno-localization of cytokinins under various auxin/cytokinin treatments revealed that the QC is also established in the ARs (Della Rovere et al., 2013). The results indicate, that an auxin maximum determined by the coordinated activity of PIN1 and LAX3, an auxin-inducible influx carrier, confines expression of the TF *WOX5* (further discussion see below) to the AR primordium tip, thereby positioning the QC. Responses to auxin application indicate that high auxin level promoted a PIN1-mediated lateral efflux from the vasculature towards the founder cells. During the later phase, *LAX3* was particularly expressed in the cells adjacent to the protruding AR primordium which points towards a function of this influx transporter in AR emergence. The results further indicate that cytokinins fine-tune the canalization and maximization of auxin via negatively affecting *PIN1* and *LAX3* expression. The whole process is further dependent on the activity of *AUX1* as other influx carrier (Della Rovere et al., 2015).

As a whole, these results support the conception that in cuttings auxin acts also as an initial signal to trigger a self-regulatory process of auxin canalization and maximization towards responding founder cells (Figure 6). During the later phases, AR primordia differentiation and AR growth is supported by a qualitative and quantitative change in auxin transport, enhanced catabolism e.g. via function of peroxidases and using the oxidation pathway and enhanced conjugation e.g. via activity of GH3 proteins (Staswick et al., 2005; Osterc and Stampar, 2011; **Ahkami et al., 2013**; Della Rovere et al., 2013; **Druege et al., 2014**; Della Rovere et al., 2015; Osterc et al., 2016).

Concept 9: Regulation of auxin signalling at levels of Aux/IAA genes, ARFs, and SAURs indicate phase-specific functions.

The concept that regulation of auxin signalling at levels of Aux/IAA proteins and ARFs has important functions in auxin-mediated guiding through the phases of AR formation in cuttings

(**Druege et al., 2014**) is supported by other recent studies. In *Arabidopsis*, ARF6 and ARF8 have been identified as positive and ARF17 as negative regulators of de-etiolation-induced AR formation in intact hypocotyls (Gutierrez et al., 2012). According to this model, Ruedell et al. (2015) found that in cuttings of *Eucalyptus globulus* homologues of *ARF6* and *ARF8* were induced during the root formation phase. *ARF8* expression was higher in far-red light acclimated cuttings that showed enhanced expression of *PIN* genes during the induction phase and enhanced AR formation when compared to white-light acclimated cuttings. In the hypocotyl of de-rooted seedlings of mung bean, seven genes encoding putative *ARFs* were down-regulated during the induction and root initiation phase (6 - 24 hpe) (Li et al., 2015a). In the same study, differential phase-dependent expression of eight auxin-responsive protein genes of the Aux/IAA-type was observed. In carnation, expression of genes encoding putative ARFs was regulated during AR formation but auxin application did not modify the expression (Villacorta-Martin et al., 2015). By contrast, several genes encoding putative Aux/IAA co-repressors were found differentially regulated during the early stages of AR formation (23 and 29 hpe), and to be auxin responsive and differentially expressed between a good- and a poor-rooting cultivar. When Abu-Abied et al. (2014) compared juvenile with mature cuttings *of E. grandis* which show high versus low rooting response to auxin application, only juvenile cuttings responded to auxin application with enhanced expression of *IAA19*, an Aux/IAA repressor. A recent study of De Almeida et al. (2015) provided new insights into the involvement of tissue-specific hormone homeostasis and signalling in rooting recalcitrance of *Eucalyptus globulus* cuttings when compared with non-recalcitrant *E. grandis*. Application of IAA during the induction phase to a great part rescued AR formation in the recalcitrant species. Auxin immunolocalization revealed that *E. grandis* accumulated more auxin at 48 hpe in the cambium zone as founder tissue of ARs than *E. globulus*, while IAA application enhanced auxin to similar levels in both species. Analysis of cambium specific gene expression by use of laser capture microdissection pointed the attention to the Aux/IAA-protein encoding gene *IAA12/Bodenlos* and the auxin-co-repressor gene *TPL* (De Almeida et al., 2015). Both genes and a cytokinin response regulator were higher expressed in *E. globulus* 24-48 hpe and repressed by auxin treatment. These results strongly support the importance of local auxin accumulation and of auxin-induced regulation of Aux/IAA proteins and TPL during induction of ARs in cuttings (Figure 6). It has to be considered here, that some of these regulators downstream of auxin may derive from tissues other than the rooting zone itself. Interestingly, Notaguchi et al. (2012) showed in *Arabidopsis thaliana* that transcripts of AUX/IAA proteins were generated in vascular tissues of mature leaves and transported via the phloem into the root system, there regulating lateral root formation.

SMALL AUXIN UP RNAs (SAURs) are genes, of which several have been shown to be transcriptionally induced by auxin in diverse plant species (Ren and Gray, 2015), but the knowledge on their role in root development is fragmentary. Whereas in rice overexpression of one *SAUR* isogene reduced root elongation and lateral root development (Kant et al., 2009), overexpression of two other isogenes in *Arabidopsis* increased elongation of the primary root (Markakis et al., 2013) and the number of lateral roots (Kong et al., 2013). Interestingly, in petunia, genes coding for SAUR-like proteins were strongly regulated after excision of the cuttings and the strongest shift in expression was observed between 6 hpe and 3 dpe (**Druege et al., 2014**). This suggests that specific SAUR-like proteins have also particular functions in the induction and differentiation in excision-induced AR formation (Figure 6). In auxin- treated cuttings of *Catalpa bungei,* transcripts of two *SAURs* showed divergent, but continuous

expression patterns during the whole course of AR formation (Wang et al., 2016). To date no molecular or biochemical mechanism has been elucidated to explain how SAUR proteins might regulate root development (Ren and Gray, 2015). However, considering that in the shoot SAURs probably control cell expansion via targeting PP2C.D phosphatases which act as inhibitors of plasma membrane H^+-ATPases (Ren and Gray, 2015), a similar function may also be involved in AR formation (Druege et al., 2016). Furthermore, similar to the Aux/IAA proteins, SAUR proteins are regulated also by other hormones than auxin including ethylene and JA (Nemhauser et al., 2006; Ren and Gray, 2015; Li et al., 2015b) providing a possible linkage point for hormonal crosstalk during AR formation (Druege et al., 2016, Figure 6).

As recently extensively reviewed and discussed also considering possible linkages to the other signalling pathways, there is numerous experimental support that nitric oxide (NO) and hydrogen peroxide (H_2O_2) have important functions in auxin-mediated AR formation in cuttings (Figure 6, Druege et al., 2016). Interestingly, production of both compounds can be stimulated by wounding (Garces et al., 2001; Jih et al., 2003) and by auxin while accumulation during AR formation was shown to depend on PAT (Liao et al., 2011; Yadav et al., 2011). Since arginine is a potential source for NO synthesis in plants (Freschi, 2013), the positive effect of dark exposure on AR formation (Klopotek et al., 2010), which involves a strong enhancement of arginine in cutting tissues (Zerche et al., 2016), may be mediated by NO.

Concept 10: Transcription factors of GRAS, AP2/ERF and WOX families link auxin signalling with cell division, fate determination and differentiation.

Transcription factors (TFs) of the GRAS family such as SCARECROW (SCR) and SHORTROOT (SHR), of the AINTEGUMENTA-LIKE (AIL) family belonging to the APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcriptional regulators, and WUSCHEL-related HOMEOBOX (WOX) proteins have important control functions during primary and lateral root development, linking auxin-signalling with cell specification and patterning and are also involved in feed-back regulation of local auxin homeostasis (Benjamins and Scheres, 2008; Ding and Friml, 2010; Horstman et al., 2014). Very recently, complete sequencing and assembly of the whole genomes of the two wild parents of *P. hybrida*, *P. axillaris* and *P. inflata*, allowed us to identify gene sequences of AP2/ERF and GRAS TFs in the transcriptome of *P. hybrida* 'Mitchell' during AR formation **(Ahkami et al., 2014; Druege et al., 2014**) and to analyse their expression during AR formation (Bombarely et al., 2016). This analysis showed that most of regulated *AP2-like* genes were induced during the whole process of AR formation. However, seven *AP2-like* genes were exclusively or predominantly induced during the induction phase, whereas five others were induced predominantly during the root formation phase. A significant number of *AP2-like* genes, including several *ERF* clades, were induced both by wounding in leaves and during AR formation in the stem base, suggesting that they provide an overlap with the ethylene signalling pathway (Bombarely et al., 2016). Also some of the *GRAS g*enes were expressed predominantly during the early or late phase. While two SHR-like proteins were down-regulated during the whole process of AR formation, several SCR-like proteins were induced during the induction phase (Bombarely et al., 2016). These results stay in line with increasing support in literature that GRAS and AP2/ERF TFs have also important control functions in AR formation in cuttings. GRAS TFs are obviously important factors for the better rooting capacity of juvenile compared to mature cuttings of woody plants, where they probably mediate the auxin control of cell fate in a phase-

and cell-type-dependent manner and participate in auxin distribution (Sanchez et al., 2007; Vielba et al., 2011; Abarca et al., 2014). Whereas null mutants of *SHR* and SCR showed reduced AR formation in *Arabidopsis* TLCs, in wild type plants increased SCR expression started in the founder cells of ARs and continued to be present in primordia and elongating ARs (Della Rovere et al., 2015). Concerning the AP2/ERF family, Rigal et al. (2012) showed that in poplar cuttings expression of the AIL-gene *PtAIL1* was enhanced during differentiation of root primordia and that the modified expression of this gene controlled the intensity of AR formation. The expression of one other poplar gene of the AP2/ERF family, *PtaERF003*, was induced by auxin and controlled the intensity of AR formation in cuttings while appearing to act as a broad regulator of growth rather than to master root development (Trupiano et al., 2013). Della Rovere et al. (2013) found indication that the TF WOX5 is involved in the auxin-mediated positioning of the QC during AR formation in *Arabidopsis*. In the same plant, *WOX5* is induced by auxin (Gonzali et al., 2005) and acts downstream of auxin-distribution in primary roots (Ding and Friml, 2010). Recently, Xu et al. (2015) provided evidence for a functional role of two other members of WOX-family in AR formation in poplar. Constitutive overexpression of either *PeWOX11a* or *PeWOX11b* strongly accelerated AR formation in cuttings, increased the number of ARs and further induced ectopic roots in the aerial parts of plants. The results altogether point towards an involvement of GRAS, AP2/ERF and WOX-type TFs in auxin-induced AR formation in cuttings (Figure 6). Furthermore, these TFs provide important linkage to other plant hormones (Druege et al., 2016).

Concept 11: PAT-dependent early auxin accumulation induces cyclin-dependent changes of the cell cycle and contributes to enhanced sink strength in the rooting zone.

In the stem base of petunia, the PAT-dependent auxin accumulation has a dual function: It provides the induction of root meristemoid formation and contributes to sink establishment via activation of invertases (Figure 4, **Ahkami et al., 2013**). Cyclin-dependent kinases and cyclins as their regulatory proteins are involved in auxin- and cytokinin-mediated governing of the cell cycle (Hartig and Beck, 2006; Komaki and Sugimoto, 2012). Expression of cyclin genes has already been related to auxin-induced AR formation in *Pinus* and *Quercus* (Lindroth et al., 2001; Neves et al., 2006). In petunia cuttings, transcripts coding for Cyclin B1 and Cyclin D accumulated from 2 dpe and 4 dpe onwards in the stem base, respectively (Ahkami et al., 2009, 2014). Also in cuttings of carnation, a number of genes encoding mitotic A-type and B-type cyclins were up-regulated from 24 hpin onwards and their expression was enhanced by auxin application (Villacorta-Martin et al., 2015). In the hypocotyl of de-rooted seedlings of mung bean, almost a hundred genes related to cell cycle were up-regulated at 24 hpe which was 24 hours before AR primordia were formed and 72 hours before ARs emerged (Li et al., 2015a). Further considering, that in the pericycle of *Arabidopsis* hypocotyl cuttings pCYCB1::GUS expression was detected after the pGH3-2::GUS expression (Sukumar et al., 2013), Druege et al. (2016) proposed the transcription of cyclin genes as important regulative factor mediating the auxin control of AR formation in cuttings via the cell cycle (Figure 6). There is strong indication in literature, that auxin action on AR formation in cuttings further involves microtubule and cell wall remodelling (Figure 6, Druege et al., 2016 and references therein)

The finding of **Agulló-Antón et al. (2011)** and **Ahkami et al. (2013**) on carnation and petunia cuttings, that auxin accumulation also contributes to sink establishment in the rooting zone via activation of sucrolytic enzymes (Figure 4), is supported by other recent studies. Treatment of cut

flowers of rose with the auxin α-napthyl acetic acid caused a transient rise in activities of vacuolar and cell wall invertase in the petals (Horibe et al., 2013). Auxin application to carnation cuttings increased the activities of all three types of invertase and also of sucrose synthase in the stem base between 24 and 72 hpin (Agulló-Antón et al., 2014). Treatment of stock plants of *Eucalyptus globulus* with far-red light, which enhanced AR formation in excised cuttings, not only stimulated expression of three genes putatively controlling auxin biosynthesis (*YUC3*) and auxin efflux (*PIN1, PIN2*) in cuttings during the induction phase but further induced the expression of two genes putatively encoding a sucrose synthase and sucrose transporter during the root formation phase (Ruedell et al., 2015). Thus, the control of sugar metabolism in the rooting zone seems to be an important function of auxin in cuttings (Figure 6).

Concept 12: Plant hormone action and sugar signalling interact during the control of AR formation and leaf senescence in cuttings.

Considering the obvious dependence of AR formation and leaf senescence in cuttings on sugars and related metabolism (**Druege et al., 2004**; **Rapaka et al., 2005**; **Ahkami et al.,** 2009, **2013,** 2014; **Klopotek et al., 2010, 2016**) and the great support in literature for sugar signalling during plant development (Lastdrager et al., 2014), it can be expected that the sugar effects involve signal functions. Furthermore, **Agulló-Antón et al. (2011)** suggested sugar-auxin crosstalk to control light-mediated AR formation in carnation (Figure 3). Since the sugar-hormone crosstalk is an important factor in the regulation of plant development (Ljung et al., 2015) it can be expected to be involved also in the control of AR formation and leaf senescence in cuttings.

During the recent years, the conserved glucose-sensing hexokinase (HXK) pathway, the SNF1-related Protein Kinase 1 (SnRK1), and the trehalose-6-phosphate (T6P) signal have been identified as important regulatory components that are interlinked and mediate effects of carbon nutrient status on plant growth and development, while they further interact with the target of Rapamycin (TOR) kinase pathway and the C/S1 bZIP TF network (Leon and Sheen, 2003; Rolland et al., 2006; Smeekens et al., 2010; Lastdrager et al., 2014). Interestingly, in the stem base of petunia cuttings several genes encoding HXK, one *SnRK1* homolog and two genes encoding one trehalose 6-phosphate synthase (TPS) and one trehalose-6-phosphate phosphatase (TPP) protein were induced at 6 hpe during the induction phase when the new carbohydrate sink was established (Ahkami et al., 2014). Generally, SnRK1 activity is repressed when sufficient sugar is available but, depending on the tissue or developmental phase studied, sucrose might have a SnRK1-stimulating role as well (Baena-Gonzalez, 2010; Lastdrager et al., 2014). There is indication in the literature that SnRK1 can mediate cell cycle progression and also affects phase transitions during plant development, while interacting with cyclin-dependent kinases (Lastdrager et al., 2014). TPS catalyses the synthesis of T6P from glucose-6-phosphate and UDP glucose downstream of glucose. Thus, the T6P pool often correlates with sugar, particularly sucrose availability (Smeekens et al., 2010; Lastdrager et al., 2014) but is further controlled by TPP-catalysed de-phosphorylation (Eastmond et al., 2003). On the one hand, T6P inhibits SnRK1 activity (Smeekens et al., 2010). On the other hand, specific members of the TPS gene family which are apparently catalytically inactive, showed sugar-dependent expression patterns while the AtTPS1 protein has been shown to interact with the cell cycle-dependent kinase CDKA1 and the kinesin KCA1, suggesting its involvement in cell cycle regulation (Smeekens et al., 2010 and references therein). Considering their observed regulation during AR induction (Ahkami et al.,

2014) and their functions in plant development particularly cell cycle, the HXK, SnRK1 and T6P-related signalling pathways can be expected to exert important functions in mediating sugar signals during the early AR induction and sink establishment phase in cuttings.

Considering possible sugar-hormone relationships during AR formation, **Agulló-Antón et al. (2011)** suggested antagonistic actions of glucose on auxin signalling (Mandadi et al., 2009; Mishra et al., 2009), which may involve the HXK pathway (Karve and Moore, 2009), as possible inhibitory function of surplus sugar levels on light-mediated AR formation in carnation (Figure 3). Interestingly, particularly glucose and auxin are considered as important players in the control of the cell cycle involving specific actions on cyclins and cyclin-dependent kinases and also of sink cell expansion (Wang and Ruan, 2013). However, not only antagonistic but also agonistic functions of sugars on auxin signalling have been found (Mishra et al., 2009; Ljung et al., 2015). Furthermore, sugars have repeatedly reported to stimulate expression of genes controlling auxin biosynthesis such as *YUCCA* and enhance auxin levels more in roots than in shoots suggesting sugars may impact auxin transport and/or conjugation pathways. In this context, phytochrome-interacting factors seem to be involved as sugar sensors both upstream and downstream of auxin (Ljung et al., 2015). There is further indication that TOR is an important linkage point for auxin and nutrient signalling via modification of protein translation (Bögre et al., 2013; Schepetilnikov et al., 2013).

Sugars may also act on AR formation via modification of the cytokinin pathway. The finding that the shoot meristem in cytokinin receptor mutants of *Arabidopsis* could be partially restored by sucrose-containing medium (Skylar et al., 2011) and others indicate that sugars and cytokinins interact in the control of meristem identity (Eveland and Jackson, 2012). In *Arabidopsis* seedlings, glucose affected the expression of genes controlling cytokinin biosynthesis and also the cytokinin response of transcription of 74% of cytokinin-regulated genes at the whole genome level, while genes controlling plant development and stress response were particularly involved (Kushwah and Laxmi, 2014). In the same study, high concentrations of glucose reduced the inhibitory effect of high cytokinin concentrations on root growth.

The negative correlations found between sugar levels in tissues particularly leaves of pelargonium cuttings at time of planting and subsequent leaf senescence and decay of cutting leaves in response to dark storage (**Druege et al., 2004**; **Druege and Kadner, 2008**) suggest that higher carbohydrate levels repress dark-induced leaf senescence in pelargonium cuttings. In this context, leaf carbohydrate levels appear as early responding indicators of carbohydrate depletion, while stem carbohydrates are more stable and reflect a of more severe carbohydrate depletion in cuttings (**Druege et al., 2004**; **Rapaka et al., 2005**; **Druege and Kadner, 2008**). The role of sugars in plant senescence is controversial since both, sugar depletion as a well as sugar accumulation have been linked to senescence (Wingler et al., 2006; van Doorn, 2008; Wingler and Roitsch, 2008). However, there is indication from studies with *Arabidopsis thaliana* that enhanced sugar supply can suppress senescence in plants, particularly when induced by darkness and/or sugar starvation. Thus, dark-induced activation of a promoter of the senescence-associated gene *sen1* in leaves which was associated with sugar depletion could be highly suppressed by the addition of sucrose, glucose or fructose at physiological concentrations (Chung et al., 1997). In another study, the accumulation of five other cDNA clones associated with leaf senescence under darkness or when photosynthesis was chemically inhibited could be suppressed by the application of sucrose (Fujiki et al., 2001). Recently, early leaf senescence

typical for the *egy1* deletion mutant defective in biosynthesis of the Ethylene-dependent gravitropism-deficient and yellow-green 1 protein, was partially restored after exogenously applied glucose while the data further indicated that the HXK pathway is involved (Chen et al., 2016). Interestingly, glucose has been shown to act antagonistically on the ethylene signalling pathway involving the HXK pathway while also repression of ABA biosynthetic genes may be involved (Rolland et al., 2006; Eveland and Jackson, 2012). For example, Yanagisawa et al. (2003) demonstrated that glucose enhances the degradation of the ethylene-insensitive 3 (EIN3), a positive transcriptional regulator in ethylene signalling. Comparing developmental and dark/starvation-induced senescence in *Arabidopsis*, Buchanan-Wollaston et al. (2005) found distinct gene expression patterns characterizing dark-induced senescence, which were similar to patterns observed during sucrose starvation-induced senescence in cell suspension cultures and pointed to an important role of JA/ethylene signalling pathways. In cut carnation (*D. caryophyllus*) flowers, sucrose treatment not only considerably delayed visible senescence, but also prevented up-regulation of the *EIN3-like* gene *Dc-EIL3* (Hoeberichts et al., 2007). During differentiation of epidermal cells in cotyledons of *Vicia narbonensis,* glucose down-regulated the expression of ethylene biosynthetic genes and an *EIN3-like* gene (Andriunas et al., 2011).

There is indeed indication that high leaf sugar levels in cuttings can counteract ethylene-mediated senescence in cuttings. Rapaka et al. (2007a, b) studied the interaction between the time of day cuttings were excised from the stock plants, carbohydrate levels in cutting tissues, and leaf senescence after short-term storage of *Portulaca* and *Lantana* cuttings in response to blocking of ethylene perception by MCP. Inhibition of ethylene perception suppressed leaf abscission regardless when cuttings were harvested. However, without MCP the numbers of abscised leaves were higher when cuttings were excised in the early morning than at later times of the day, while they were negatively correlated with leaf sugar levels at harvest. The authors concluded that the inhibitory effect of high sugar levels on leaf abscission is mediated by a suppressive effect of sugars on ethylene signalling (Rapaka et al., 2007a, 2007b). A following study further supported an ambivalent role of ethylene action in pelargonium cuttings in dependence on the carbohydrate availability. Established relationships between carbohydrate levels, ethylene production and ethylene responses of leaf senescence and AR formation in stored cuttings of two cultivars under the influence of varied light intensity before cutting harvest support the conclusion that ethylene signalling accelerates leaf senescence when carbohydrate levels in the tissue is low but promotes AR formation in the stem base under conditions of high carbohydrate availability (Rapaka et al., 2008).

In summary, there is increasing support in the literature suggesting that sugars may act on AR formation and leaf senescence in cuttings as signals and via crosstalk to plant hormone pathways. However, functional analysis of putative sugar and hormone signalling cascades including dose response studies of AR formation and leaf senescence to sugars and hormones is necessary to unravel these relationships.

4.5 Conclusion

Based on the investigations summarized, compiled and discussed in the context of current literature in chapters 2, 3 and 4, respectively, AR formation and leaf senescence in shoot tip

cuttings is the outcome of a complex interaction between plant hormone related pathways and a metabolic response which is summarized in Figure 7.

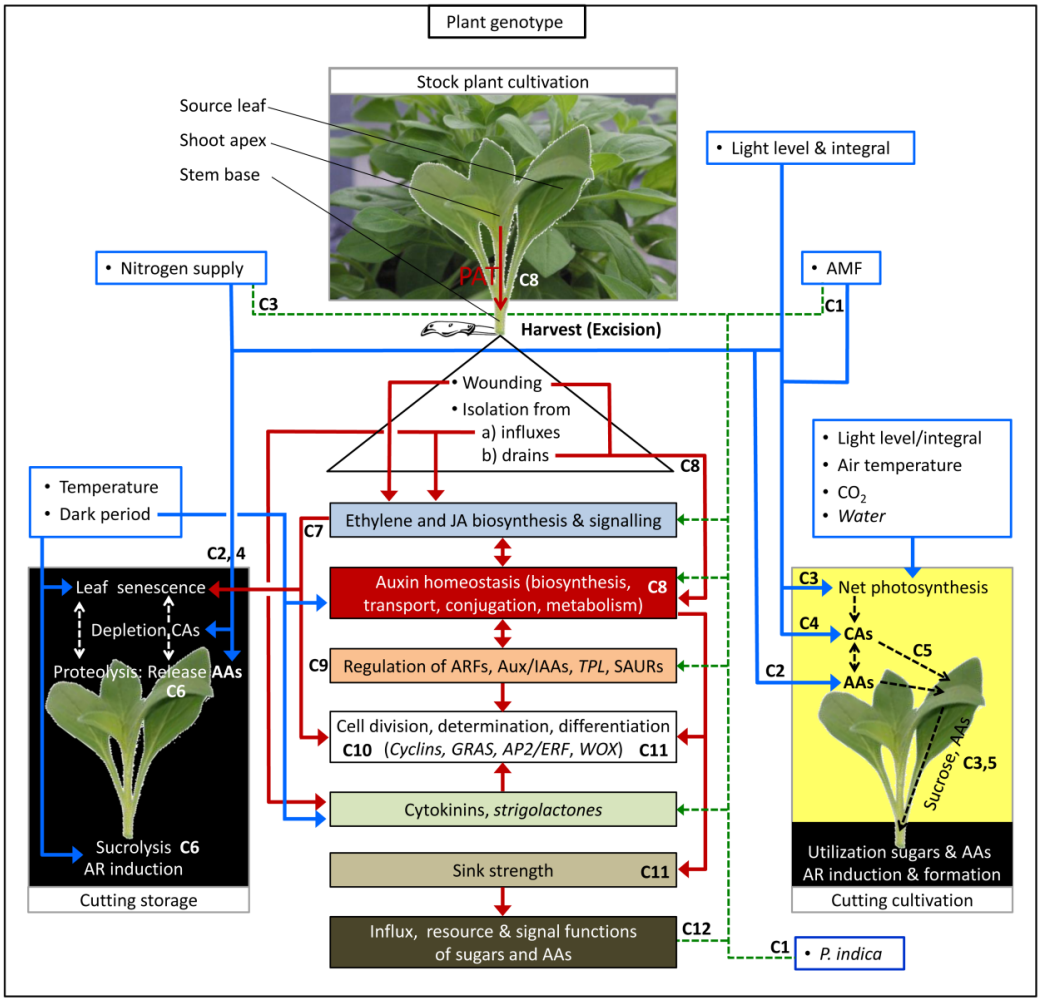

Figure 7 Summarizing model of factors and processes controlling AR formation and leaf senescence in ornamental shoot tip cuttings based on the publications summarized in chapter 2 and on related publications discussed in chapter 4 (in this case presented in italic letters), using petunia as an example. Plant genotype provides the basic response capacity at stock plant and cutting level. Source leaves, the growing shoot apex and the stem base as rooting zone are marked as important physiological units of the source-sink network of the cutting. The left and right windows focus on cuttings which experience dark storage and immediate cultivation, respectively. Influences of environmental factors at stock plant level and during storage and cultivation of cuttings are presented in blue-framed boxes, with blue arrows targeting important physiological components or processes mediating the developmental response. Red arrows mark directive linkages between cutting excision, plant hormone homeostasis and signalling and controlled cellular functions. Green dashed arrows show hypothetical linkages between nitrogen supply and infection with AMF or *P. indica*, respectively, and plant hormone related pathways. C1–C12 indicate the relevance of components and pathways to the Concepts 1–12 of the discussion chapter. CAs = carbohydrates, AAs = amino acids

Fully developed leaves, the shoot apex and the stem base as rooting zone constitute important functional units as source organs, already existing carbohydrate utilisation sink and newly developing sink organs, respectively. Wounding at the cutting site and isolation from the network of signal and resource fluxes triggers a hormonal response at the levels of homeostasis and signalling. Wounding and probably other stresses deriving from the cut influx of water and minerals trigger ethylene and JA biosynthesis and signalling. In case of sensitive plant species, action of ethylene and possibly also of JA can induce leaf senescence. This particularly applies to conditions of dark storage which allow for accumulation of ethylene in the storage containers and cause depletion of sugars as potential antagonists of ethylene signalling. At the same time, accumulation and signal perception of ethylene and JA in the stem base contribute to formation of ARs. The isolation of the cutting from the basipetal auxin drain causes a PAT-dependent accumulation of IAA in the stem base. This probably initiates self-regulation of auxin homeostasis and auxin signalling to provide induction of founder cells and cyclin-dependent subsequent initiation and further differentiation of ARs. Furthermore, the PAT-dependent auxin accumulation contributes to sink establishment via activation of sucrolytic enzymes such as invertases, thereby enhancing the influx of assimilates and co-transported amino acids in the stem base where they act as resources and probably also as signals to feed and regulate AR formation. A depletion of cytokinins and strigolactones after the cut from the root system further contribute to AR induction. ARFs, Aux/IAA proteins, TPL and SAUR proteins and TFs of the GRAS, AP2/ERF and WOX families are important regulative components of auxin signalling and function, while they further provide linkages for crosstalk with other hormones. In particular, crosstalk between auxin and ethylene in both directions and fine-tuning of auxin transport by cytokinins can be expected.

The level of supplied nitrogen to stock plants limits the abundance of organic nitrogen in the cuttings and thus the availability of amino acids to be transported from source leaves to the stem base. The stimulation of AR formation by high nitrogen supply and content may further involve stimulation of photosynthesis in and sucrose export from source leaves and modification of plant hormone homeostasis and signalling. However, the high nitrogen supply causes shortage of carbohydrates in the cuttings, resulting in pronounced sugar depletion during dark storage. Depletion of carbohydrates during dark storage reduces the intensity of AR formation under conditions of low photosynthesis of cuttings after planting. Then the low carbohydrate levels after dark storage particular in the source leaves limit the influx of carbon via sucrose and of co-transported amino acids in the stem base.

Net photosynthesis of cuttings has a strong impact on AR formation via feeding the sucrose export from source leaves and is dependent on the plant genotype, the acclimation of cuttings to the environmental conditions during cutting production and the environmental conditions during cultivation of the cuttings. When photosynthesis of cuttings is high, dark storage of cuttings can advance AR formation via the rise in sucrolytic activity in the stem base enhancing the sink strength relative to the shoot apex and via AR induction leading to meristemoid formation. Obviously, auxin transport and enhanced auxin:cytokinin ratios in the stem base contribute to these dark responses. When the dark stored cuttings are planted thereafter, high photosynthesis allows for enhanced transport of sucrose and co-transported amino acids towards the developing ARs. Progressive depletion of carbohydrates during dark exposure promotes proteolysis in cutting leaves, which increases the abundance of amino acids in the leaf and the rooting zone. In case of high photosynthesis of cuttings counteracting the carbohydrate depletion after the dark

phase, mobilization of nitrogen and enhancement of amino acid levels can contribute to the dark-induced enhanced AR formation.

Infection of stock plants with AMF can reduce dark storage-induced carbohydrate shortage in cuttings while enhanced carbohydrate reserves at time of harvest and enhanced photosynthesis may be involved. Beneficial effects of AMF in stock plants may further involve plant hormones. Action of plant hormones and particularly of auxin seems to be particularly involved in the strong stimulation of AR formation when *P. indica* is supplied to the rooting substrate.

4.6 Outlook

The investigations of this thesis have highlighted the influences of environmental key factors during the propagation chain on AR formation and leaf senescence in ornamental shoot tip cuttings and provided insight into the dynamic responses of cuttings at the levels of primary metabolism and plant hormone related pathways. Using petunia as model and combination of metabolic studies with specific and broad analysis of the transcriptome pointed to an important role of the carbohydrate source-sink relationships and of auxin homeostasis and signalling as master control box of AR formation. Systematic analysis of different cutting parts highlighted the importance of a functional equilibrium of the whole cutting, with the shoot apex, source leaves and the rooting zone as important interdependent functional units. Further, pharmacological approaches provided evidence for the functional roles of ethylene biosynthesis and perception in AR formation and leaf senescence and of PAT and auxin homeostasis in AR induction and carbohydrate metabolism in cuttings.

Future research should follow three directions, which need to be integrated for a better understanding of important bottlenecks of AR formation in cuttings under the influence of global young plant production chains.

1. High resolution and functional analysis of key processes in the rooting zone of petunia cuttings

In the first perspective, the relevant processes in the stem base of cuttings should be further analysed at high resolution addressing the different tissues and cells involved and localizing the molecular factors and processes of interest. Considering the emerging methods available for molecular analysis of petunia (Bombarely et al., 2016; Vandenbussche et al., 2016) and the importance of AR formation for clonal propagation of this species, *P. hybrida* is the model of choice for such future investigations.

With regard to the obvious dependence of AR formation on sugars and carbohydrate metabolism (**Rapaka et al., 2005**; **Druege and Kadner, 2008**; Ahkami et al., 2009, 2014; **Klopotek et al., 2016**) and the still unknown signal functions of sugars in this context, sugar signal effects and sugar-hormone crosstalk in the rooting zone should be analysed in detail. Sterile *in vitro* rooting systems already employed in studies on other plant species (Correa et al., 2005) are ideal for such investigations because sugars and hormones can be easily applied phase-specifically without promoting development of saprophytes. The expression of genes putatively controlling the respective signalling pathways should be analysed together with the development of ARs,

followed by addressing the function of genes (see further below). Because the function of strigolactones in AR formation is unknown for petunia and obviously depends on plant species, the AR inducing principle and environmental conditions (Rasmussen et al., 2012; Sun et al., 2015; Urquhart et al., 2015), petunia mutants of strigolactone biosynthesis, transport and perception already available in the scientific community (Kretzschmar et al., 2012; Seto and Yamaguchi, 2014) should be used for detailed analysis of the strigolactone function during specific phases of AR development.

Considering that root development can be regulated post-transcriptionally at protein level (Mattei et al., 2013; Stauffer and Maizel, 2014), proteome analysis should complement the transcriptome and metabolome approaches. Further, laser microdissection, immunolocalization or reporter-promotor constructs (De Almeida et al., 2015; Della Rovere et al., 2015) should be used to assign the interesting compounds such as hormones and the expression of genes of interest to particular tissues, cells or even subcellular structures. Reporter genes for ß-glucuronidase (GUS), the green fluorescent (GFP) protein or the yellow fluorescent protein (YFP) can be fused to the promotor of the gene under focus (Ulmasov et al., 1997; Nagai et al., 2002; Heisler et al., 2005; Voss et al., 2013). For localization of the auxin response, the *DR5* promotor, which has been generated by mutation of the auxin responsive element in the *GH3* promotor of soybean (Ulmasov et al., 1997), has already been frequently used in studies on *Arabidopsis*. In a current project, we use transgenic petunia encoding *DR5-GUS*, *-GFP* and *-YFP* constructs to analyse the auxin response under the influence of nitrogen conditioning and dark exposure of cuttings. However, it has to be considered that the DR5 reporting system involves the action of the whole auxin signalling cascade including ARFs and Aux/IAA proteins, the expression of which is strongly modified during AR formation (**Druege et al., 2014**). In *Arabidopsis*, the DII-Venus sensor has recently been developed as interesting alternative auxin sensing system (Brunoud et al., 2012). This sensor system uses a fast maturating form of YFP, which however is under the direct control of the TIR1/AFB complex because it is fused to the auxin-interaction domain (termed DII) of Aux/IAA proteins. As a result, the signal of this reporting system is closely linked to the auxin concentration in the tissue (Brunoud et al., 2012). Thus, DII-Venus appears as promising tool for future investigations in petunia. Lab-on-a-chip or microfluidic technologies using biosensors have emerged during recent years (Jones et al., 2013; Stanley et al., 2016) and already been successfully employed to analyse the dynamics of sugar metabolism in intact roots of *Arabidopsis* (Grossmann et al., 2011). These tools should provide interesting solutions in the future for non-invasive real-time analysis of processes controlling AR formation, possibly even in petunia.

The function of candidate genes putatively controlling AR formation either in general (**Druege et al., 2014**) or under the particular influence of a specific environmental factor (**Klopotek et al., 2016**), should be further analysed by using mutants or transgenic plants with modified transcription of target genes. The transposon insertion line W138 provides excellent perspectives for the analysis of gene knock-outs in petunia (Vandenbussche et al., 2016). Circa 400.000 individuals from this line have already been sequence-indexed for *dTph1* insertions, so that plants with insertions in candidate genes can easily be identified *in silico* (Vandenbussche, pers. communication 15 October 2015). Because transformation protocols are available for *P. hybrida* (Mussmann et al., 2011) transgenic lines through sense or anti-sense approaches should also be involved. This would also allow assignment of gene functions to specific tissues and rooting

phases by use of tissue-specific, phase-specific or inducible promotors for the corresponding RNAi constructs.

The sequencing, assembly and annotation of the complete genomes of *P. axillaris* and *P. inflata* as the two wild parents of *P. hybrida* (Bombarely et al., 2016) provides an excellent new basis for both, the detection of new candidate genes and analysis of gene function. A virtual transcriptome can be generated *in silico* from the genome. With the help of this data, all interesting sequences on the microarray (**Druege et al., 2014**) which are based on ESTs (Breuillin et al., 2010) can be assigned to the complete genes, which also include sequences which could previously not annotated. In future transcriptome studies, the virtual transcriptome should be combined with RNAseq technology, which provides discovery and quantification of RNAs of the entire transcriptome in a single high-throughput sequencing assay (Wang et al., 2009; Conesa et al., 2016). For functional analysis, the genome data provides the important information about the number of copies of candidate genes in the genome and the structure of genes for the selection and construction of promotors and RNAi constructs. The genome data of the two parents of *P. hybrida* should further be used in segregation studies of crosses to combine the mapping of quantitative trait loci (QTL) for AR formation with the search for putatively functioning genes on or close to the identified QTL. Interestingly, *P. axillaris* and *P. inflata* contrast in the intensity of AR formation and the dark response (Druege, unpublished), which provides excellent conditions for segregations studies. Particularly but not only for such studies, automated image-based phenotyping systems which have recently been developed for carnation cuttings (Birlanga et al., 2015) should be developed as powerful tools for detailed and comprehensive analysis of the rooting phenotype.

2. Analysis of the whole response system: Cutting in the environment

Because the processes in the rooting zone of shoot tip cuttings are highly dependent on the function of source leaves **(Rapaka et al., 2005**) and stay in a competitive relationship to the carbon demand of the shoot apex (**Klopotek et al., 2016**), the whole cutting has to be considered as a complex response system. In this context, distinct physiological units of the cuttings, which have particular functions and respond specifically to environmental factors (Druege et al., 2016, Figure 7), should be integrated into a mechanistic model of the whole cutting. To move forward from simple models (e.g. Figure 2, **Klopotek et al., 2016**) to sophisticated models as currently being developed for understanding the branching of roots (Atkinson et al., 2014; Goh et al., 2014), there is the need to involve different disciplines such as mathematics and computer science.

The stress- and particularly dark-induced senescence of cutting leaves on the one hand can evoke a problem *per se* but on the other hand - obviously depending on its intensity and transitory character - can contribute to nitrogen allocation to the developing roots (Zerche et al., 2016). Future studies should follow both processes. Thus, the function of sugar-ethylene crosstalk in senescing leaves should be further analysed as well as the specific contribution of individual amino acids and other N compounds as mobile nutrients and signals. These studies should include pharmacological approaches, isotope labelling, physiological manipulations such as removal or shading of cutting parts and analysis and modification of expression key genes putatively involved. Considering the importance of dark storage for cutting propagation in

general and the increasing restrictions in the use of chemically-synthesized auxins, the contribution of different auxin sources, pathways of transport and other plant hormones to dark-stimulated AR formation should be further investigated. Furthermore, regarding the great influence of light spectrum applied to plants on plant hormone pathways (De Lucas and Prat, 2014), future investigations should also focus on the light spectrum as promising tool to enhance AR formation in cuttings.

3. Translation to other cultivars and plant species

The production of young ornamental plants has to cope with the enormous diversity of used plant species and cultivars. Thus, there is the need to translate the knowledge established for a certain cultivar or species to other important crop genotypes. Ideally, the plant characteristic which determines the respective bottleneck should be measurable as easy and fast as possible so that a large number of samples can be analysed. An example for such an approach to the nitrogen and carbohydrate source limitation of AR formation is the non-invasive analysis of N_t, other N fractions and carbohydrates in cuttings by near infrared spectroscopy (Lohr et al., 2015, 2016). Based on the found N and carbohydrate limitation of AR formation in chrysanthemum, pelargonium and poinsettia (**Druege et al., 2000, 2004**; **Rapaka et al., 2005**; **Zerche and Druege, 2009**) this method has been newly developed and in case of N fractions found to be applicable also for *Impatiens* and *Osteospermum*. In a current project, we are testing whether N and carbohydrate fractions are suitable characteristics to detect N and carbohydrate limitations of AR formation and senescence in practical young plant production chains of two industrial partners.

Considering the physiological bottlenecks of AR formation and leaf senescence discovered within the scope of the present thesis and other related studies of the author, the range of cultivars and species should be tested and categorized into response groups at physiological level. In this context, the following hypotheses may be tested for its broad application.

Dark storage-induced leaf senescence in cuttings

- is high when the plant genotype reveals a high ethylene sensitivity of leaves

The benefit of dark storage to AR formation in cuttings

- is low when the plant genotype has a low photosynthetic activity of excised shoots
- depends on the dark response of carbohydrate metabolism particularly in the stem base
- depends on the dark response of nitrogen metabolism particularly in leaves
- depends on the dark response of plant hormone pathways particularly of auxin

The benefit of *P. indica* inoculation to AR formation in cuttings

- depends on the dynamics of auxin accumulation in the stem base of the plant in relation to the auxin release by the fungus

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6. German summary – Zusammenfassung

Die Bildung von Adventivwurzeln (AW) in Stecklingen ist ein pflanzlicher Entwicklungsprozess, der für die vegetative Vermehrung vieler Zierpflanzenarten genutzt wird. Als Stecklinge finden dabei häufig die Spitzen von Seitentrieben, sogenannte Kopfstecklinge, Verwendung, die über das apikale Sprossmeristem und mindestens ein voll entwickeltes Blatt verfügen. Ausgelöst durch die Verwundung und Isolierung von der Mutterpflanze wird in bestimmten Zellen in der Sprossbasis ein neues Entwicklungsprogramm gestartet, das über mehrere Phasen zur Wurzelneubildung führt und so dem Spross das Überleben ermöglicht. In der "Induktionsphase" werden die betroffenen Zellen ohne anatomische Veränderungen so umprogrammiert, dass sie in der nachfolgenden "Wurzelbildungsphase" durch Zelldifferenzierungs- und -wachstumsprozesse eine neue Wurzel ausbilden können. Die hierauf basierende Jungpflanzenproduktion für Europa erfolgt im Rahmen einer mehrstufigen Produktionskette, die mit der außereuropäischen Stecklingsproduktion an Mutterpflanzen z.B. in Ostafrika beginnt. Nach der Ernte werden die Stecklinge je nach Pflanzenart über unterschiedlich lange Zeiträume in Kühlräumen dunkel gelagert, bevor sie nach Europa transportiert, dort in Substrat gesteckt und zur Bewurzelung in Gewächshäusern kultiviert werden. Unter diesen Bedingungen wird wiederholt eine mangelhafte Bewurzelung beobachtet. Dabei behindert das unzureichende Verständnis der endogenen und umweltabhängigen Steuerung der AW-Bildung die Optimierung der Produktionskette. Neben der Regulierung durch Phytohormone, insbesondere Auxine, ist die Versorgung des neu etablierten Wachstumszentrums mit C- und N-Quellen sowie weiteren Nährstoffen wichtig. Die beteiligten Wirkungsketten und das Zusammenspiel der Faktoren sind jedoch nur unzureichend verstanden. Die in der vorliegenden Habilitationsschrift dargestellten Arbeiten hatten zum Ziel, physiologische und molekulare Faktoren und Prozesse zu identifizieren, welche die AW-Bildung in Stecklingen kontrollieren. Wenn die Umweltbedingungen zusätzlich zur AW-Bildung die Stecklingsvitalität beeinflussten, wurde der Fokus um die Blattseneszenz und Überlebensrate der Stecklinge erweitert. Ein besonderer Schwerpunkt lag auf der Wirkung solcher Umweltfaktoren, die mögliche Ansatzpunkte für eine Optimierung der Produktionskette darstellen.

Vor dem Hintergrund bereits beschriebener allgemeiner positiver Effekte von arbuskulären Mykorrhiza (AM)-Pilzen und dem Wurzelendophyten *Piriformospora indica* auf die Entwickung und das Wachstum von Pflanzen wurden die Einflüsse dieser Mikroorganismen auf die AW-Bildung in Stecklingen untersucht. Positive Effekte einer Inokulation von Bewurzelungssubstraten mit AM-Pilzen auf die AW-Bildung in Stecklingen sind in der Literatur mehrfach beschrieben. In einem alternativen Ansatz wurde die Hypothese geprüft, ob bereits eine Inokulation der Mutterpflanzen mit AM-Pilzen die Überlebensrate und/oder AW-Bildung erhöhen kann (**Druege et al., 2006**). Die Besiedlung von *Euphorbia pulcherima* Mutterpflanzen mit dem AM-Pilz *Glomus intraradices* erhöhte die Überlebensrate der Stecklinge nach einer Dunkellagerung während der Bewurzelung unter niedrigem Lichtangebot. Darüber hinaus stimulierte die Mykorrhizierung der Mutterpflanzen die AW-Bildung in den dunkel gelagerten Stecklingen. Die stärkste Förderung der AW-Bildung durch die AM war mit besonders hohen Gesamtzuckerkonzentrationen in den Stecklingsblättern zum Erntezeitpunkt verbunden. Eine nachhaltig veränderte Source-Sink-Situation im Spross in Folge der Anpassung an die Wurzelsymbiose und häufig beschriebene hormonelle Effekte der AM wurden als mögliche Wirkungsfaktoren für die erhöhte Überlebensrate und verbesserte Bewurzelungsfähigkeit von Stecklingen mykorrhizierter Mutterpflanzen diskutiert (**Druege et al., 2006**). Der Wurzelendophyt *P. indica* stellt aufgrund

seiner im Gegensatz zu AM-Pilzen einfachen axenischen Kultur eine interessante Alternative dar, die AW-Bildung auf biologischem Weg zu stimulieren. **Druege et al. (2007**) untersuchten den Einfluss einer Inokulation des Bewurzelungssubstrates mit *P. indica* auf die AW-Bildung in Stecklingen von drei Pflanzenarten. In *Pelargonium hortorum* und *Euphorbia pulcherrima* wurden Anzahl und Länge der AWn durch den Endophyten wesentlich erhöht. Stecklinge von *Petunia hybrida* hingegen, die wesentlich schneller bewurzelten als die der beiden anderen Pflanzenarten, zeigten keine Reaktion auf *P. indica*. Die Hypothese der Autoren, dass der Endophyt über Phytohormonsignalketten insbesondere der Auxine wirkt, wird durch jüngere Publikationen zur Beteiligung von Phytohormonen bei der Stimulierung anderer Wachstums- und Entwicklungsprozesse durch *P. indica* gestützt.

Vor dem Hintergrund, dass der C- und N-Stoffwechsel in Pflanzen in enger Wechselwirkung zueinander stehen, war die Anhängigkeit der AW-Bildung in Stecklingen vom Stickstoffgehalt und den Kohlenhydratkonzentrationen in einzelnen Stecklingsorganen Gegenstand weiterer Forschungsarbeiten. An drei Pflanzenarten wurde der Einfluss der Stickstoffdüngung der Mutterpflanzen in Abhängigkeit von der Dunkellagerung der Stecklinge und den Lichtbedingungen während der Stecklingsproduktion und der Bewurzelungsphase untersucht. Eine höhere Stickstoffdüngung der Mutterpflanzen von *Chrysanthemum indicum*, *Pelargonium hortorum* und *Euphorbia pulcherrima* hatte niedrigere Stärkekonzentrationen zum Erntezeitpunkt insbesondere in den Stecklingsblättern zur Folge und führte zu einem stärkeren Abbau der Zuckerreserven während einer nachfolgenden Dunkellagerung (**Druege et al., 2000, 2004**; **Zerche and Druege, 2009**). Bei allen drei Kulturen korrelierten Anzahl und Länge der gebildeten AWn positiv mit dem N-Gehalt der Stecklinge. Als mögliche Ursachen der positiven Wirkung hoher N-Gehalte wurden eine erhöhte Verfügbarkeit und Anlieferung organischer N-Komponenten insbesondere von Aminosäuren in der Bewurzelungszone diskutiert, die durch aktuelle Ergebnisse zu *Petunia hybrida* gestützt werden (Zerche et al., 2016). Darüber hinaus wurden Stickstoffwirkungen über den Kohlenhydrattransport und die Phytohormonsignalketten diskutiert (**Druege et al., 2000, 2004**). Die Limitierung der AW-Bildung durch geringe N-Gehalte traf bei *C. indicum* und *E. pucherrima*, die bei relativ hohem Lichtangebot produziert und bewurzelt wurden, auch auf dunkel gelagerte Stecklinge zu, obwohl diese nach hoher N-Versorgung die niedrigsten Kohlenhydratkonzentrationen aufwiesen. Hingegen wurde die N-Limitierung der AW-Bildung in Pelargonienstecklingen, die unter hohem Lichtangebot produziert jedoch unter Schwachlicht bewurzelt wurden, durch eine Dunkellagerung der Stecklinge gebrochen. Die AW-Bildung korrelierte hierbei positiv mit den Zuckergehalten in den Stecklingsblättern zum Stecktermin, die wiederum die nachfolgende Zuckerakkumulation in der Sprossbasis determinierten (**Druege et al., 2004)**. Die abgeleitete Hypothese der Bedeutung der Photosynthese und des Kohlenhydratexportes für die AW-Bildung wurde dadurch gestützt, dass die AW-Bildung in *E. pulcherrima* zusätzlich zum N-Gehalt eine positive Abhängigkeit von der Saccharosekonzentration in den Blättern zum Stecktermin und von dem Tagesintegral der photosynthetisch aktiven Strahlung während der Bewurzelung aufwies (**Zerche and Druege, 2009**).

Im Folgenden untersuchten **Rapaka et al. (2005)** die Wechselwirkungen zwischen Kohlenhydratreserven und Photosynthese in Pelargonienstecklingen unter den Einflüssen einer kurzzeitigen Dunkellagerung als Transportsimulation und einem variablem Lichtangebot während der Stecklingsproduktion und der Bewurzelung. Zunehmendes Lichtangebot während der Stecklingsproduktion führte zu höheren Zuckerkonzentrationen insbesondere in der Sprossbasis,

reduzierte aber gleichzeitig das nicht-photochemische Quenching der Chlorophyllfluoreszenz (qN) der Blätter und die Bewurzelung der ungelagerten Stecklinge unter niedrigem Lichtangebot. Eine Dunkellagerung der Stecklinge vor der Bewurzelung unter dem niedrigen Lichtangebot reduzierte die AW-Bildung, wobei die Wurzelanzahl positiv mit der Saccharosekonzentration der Blätter zum Stecktermin korrelierte. Ein höheres Lichtangebot während der Bewurzelung jedoch eliminierte die lager-bedingte Beeinträchtigung der AW-Bildung und entkoppelte die Beziehung zwsichen den Blattzuckern zum Stecktermin und der Wurzelanzahl. Die Gesamtvariabilität der Wurzelanzahl aller Behandlungen wies eine positive Korrelation zu den durchschnittlichen Saccharosekonzentrationen in den Stecklingsblättern während der ersten Bewurzelungswoche auf. **Rapaka et al. (2005)** schlussfolgerten hieraus, dass ein hoher Kohlenhydratexport der Source-Blätter während der Bewurzelung eine wesentliche Voraussetzung für eine intensive AW-Bildung in der Sprossbasis ist und dieser eine Funktion der Reserven zu Bewurzelungsbeginn und der lichtabhängigen Photosynthese während der Bewurzelung darstellt. Nachfolgende Untersuchungen von **Druege und Kadner (2008)** zum Einfluss der Bewurzelungstemperatur auf Kohlenhydratkonzentrationen und AW-Bildung in zuvor dunkel gelagerten, d. h. an Kohlenhydraten verarmten Pelargonienstecklingen, unter Niedriglichtbedingungen erhärteten diese Hypothese. So korrelierte eine erhöhte Anzahl gebildeter AWn in Folge einer reduzierten Luftemperatur positiv mit der durchschnittlichen Saccharosekonzentration insbesondere in den Stecklingsblättern während der Bewurzelung. Eine höhere Photosyntheserate der Blätter unter der niedrigen Lufttemperatur deutete darauf hin, dass die niedrige Temperatur über eine reduzierte Respiration die Kohlenstoffbilanz erhöhte, so dass ein höherer Saccharosepool in den Blättern für den Export in Richtung Sprossbasis zur Verfügung stand (**Druege and Kadner, 2008**).

Nach Etablierung von *Petunia hybrida* als neues Modellsystem und der Charakterisierung metabolischer Phasen der AW-Bildung in Stecklingen (Ahkami et al., 2009) untersuchten **Klopotek et al. (2010**) den Einfluss einer Dunkellagerung auf die AW-Bildung und deren Beziehungen zum Kohlenhydrathaushalt. Eine einwöchige Dunkellagerung der Petuniensteckline bei 10 °C oder 20 °C führte zu einer starken Förderung der AW-Bildung während der nachfolgenden Kultur der Stecklinge unter Licht. Anatomische Untersuchungen zeigten, dass während einer kühlen Dunkellagerung bereits erste neue meristematische Zellen gebildet werden und die nachfolgende Differenzierung der Wurzeln in der Lichtphase wesentlich schneller erfolgt als bei direkt gesteckten Stecklingen. Die Dunkellagerung führte zu einer Kohlenhydratverarmung bevorzugt in den Blättern, jedoch akkumulierten in der Sprossbasis während der nachfolgenden Lichtperiode höhere Zuckerkonzentrationen als bei direkt gesteckten Stecklingen. Messungen des CO_2-Gaswechsels zeigten, dass Petuniensteckline im Vergleich zu Pelargonienstecklingen eine höhere Nettophotosyntheserate aufweisen, die durch eine vorherige Dunkellagerung nicht beeinflusst wird (**Klopotek et al., 2012, 2016**). Jedoch akkumulierten zuvor dunkel gelagerte Petunien-Stecklinge unter Lichtbedingungen bei gleicher Gesamttrockenmasseproduktion einen wesentlich höheren Anteil der Trockenmasse in den Wurzeln als direkt gesteckte Stecklinge (**Klopotek et al., 2016**). Die offenbar erhöhte Sinkaktivität der Bewurzelungszone nach Dunkellagerung korrelierte mit einer organspezifischen Induktion und Aktivierung von Invertasen. In einem abgeleiteten Modell stellen die Sprossbasis-spezifische Aktivierung der Zellwandinvertasen in der Dunkelphase und die Photosynthese der Blätter in der anschließenden Lichtphase wichtige den Dunkellagereffekt determinierende Sink- und Source-Komponenten dar (**Klopotek et al., 2016**). Ausgehend von vorangegangenen Arbeiten, die eine Förderung der AW-Bildung in *Dianthus caryophyllus* durch eine kühle Dunkellagerung

beschrieben hatten, stellten **Agulló-Antón et al. (2011)** die Wechselwirkungen zwischen Licht, Kohlenhydraten und dem Phytohormon Auxin in den Mittelpunkt ihrer Untersuchungen. Eine schwache Belichtung der Stecklinge während der Lagerung führte zu einem starken Anstieg der Zuckerkonzentrationen insbesondere in der Sprossbasis, reduzierte dort jedoch die Konzentration des Auxins Indol-3-essigsäure (IAA), das Verhältnis von IAA und dem Cytokinin Zeatin und die AW-Bildung im Vergleich zu dunkel gelagerten Stecklingen. Eine Pulsapplikation mit Auxin vor dem Stecken förderte die AW-Bildung insbesondere in den ungelagerten, weniger in den hell gelagerten und nur geringfügig in den dunkel gelagerten Stecklingen. Basierend auf diesen Ergebnissen entwickelten **Agulló-Antón et al. (2011**) ein Konzept zur Auxin-Cytokinin-Zucker Wechselwirkung in der Sprossbasis auf die AW-Bildung von Nelkenstecklingen. Eine Auxinakkumulation während der Dunkellagerung wird als induzierendes Prinzip für die lager-stimulierte AW-Bildung vermutet, dem eine Belichtung über eine Reduktion der Auxinkonzentration sowie die Generierung supra-optimaler Zucker- und Cytokininkonzentrationen entgegen wirkt.

Vor dem Hintergrund der von anderen Autoren bereits beschriebenen Funktion des Ethylen als Signal, das in empfindlichen Pflanzenarten eine Blattseneszenz auslösen kann, und einer bereits vorgenommenen Charakterisierung der verwundungs- und temperaturabhängigen Ethylenproduktion in Pelargonienstecklingen (Kadner et al., 2000) untersuchten **Kadner and Druege (2004)** den Beitrag der Ethylenperzeption bei der durch Dunkellagerung induzierten Blattseneszenz in Pelargonienstecklingen. Eine Applikation von 1-Methlycyclopropen (MCP) oder zwei Silbersalzen als Blocker der Ethylenperzeption vor der Dunkellagerung reduzierte eine lager-induzierte Vergilbung der Stecklingsblätter um etwa 50 %, verminderte jedoch auch die AW-Bildung. Eine gleichzeitig hohe Steigerung der Ethylenproduktion der Stecklinge durch die Ethylenblocker deutete darauf hin, dass die Ethylenperzeption in den Pelargonienstecklingen einen wichtigen Prozess nicht nur für die Auslösung der Blattseneszenz und die Stimulierung der AW-Bildung darstellt, sondern darüber hinaus auch für eine negative Rückkopplung der wund-induzierten Ethylenbiosynthese (**Kadner and Druege, 2004**).

Obwohl Auxin als wichtiger Induktor der AW-Bildung akzeptiert ist, waren bis vor Kurzem die regulierenden Prozesse der Auxinhomöostase und -signalketten sowie die dadurch kontrollierten cytologischen und metabolischen Prozesse während der AW-Bildung in Stecklingen nahezu unbekannt. Durch Einsatz von Naphthylphthalaminsäure (NPA) als Blocker des polaren Auxintransportes (PAT) untersuchten wir den funktionellen Beitrag des PAT für die Auxinhomöostase und die cytologischen sowie metabolischen Prozesse in der Sprossbasis von *P. hybrida* (**Ahkami et al., 2013**). Eine Sprühbehandlung der Stecklinge mit NPA unmittelbar nach der Ernte verhinderte einen 24h-Peak der IAA-Konzentration, reduzierte einen Anstieg der Aktivitäten der Zellwand- und vakuolären Invertase während der Induktionsphase und verhinderte nahezu vollständig die AW-Bildung. Die Induktion der ersten Zellteilungen des neuen Wurzelmeristems und die Etablierung des neuen Kohlenhydratsinks in der Sprossbasis werden als zwei wesentliche Wirkungen des PAT und der frühen Auxinakkumulation angesehen (**Ahkami et al., 2013**). Mithilfe eines für *P. hybrida* entwickelten Microarrays analysierten **Druege et al. (2014)** das Transkriptom der Gene mit kontrollierenden Funktionen für die Phytohormonhomöostase und -signalketten in der Sprossbasis während der AW-Bildung. Eine große Anzahl von Genen von Aminocyclopropancarbonsäure (ACC)-Synthase und -Oxidase als wichtige Enzyme der Ethylenbiosynthese zeigten einen starken Anstieg der Expression über alle

Phasen der AW-Bildung. Unter weiterer Berücksichtigung, dass viele Gene für Ethylen-responsive Transkriptionsfaktoren ein ähnliches Expressionsmuster aufwiesen und die Anzahl gebildeter AWn in entwurzelten Sämlingen von *P. hybrida* durch Applikation von Inhibitoren der Ethylenbiosynthese und -perzeption vermindert, durch ACC jedoch gefördert wurde, schlussfolgerten **Druege et al. (2014)**, dass Ethylen eine stimulierende Wirkung auf die AW-Bildung in *P. hybrida* hat. Die Stecklingsverwundung, weitere Stressfaktoren in Folge der Abtrennung von der Wasser- und Mineralstoffversorgung sowie die frühe Auxinakkumulation wurden als wesentliche die Ethylensignalkette stimulierende Prinzipien diskutiert. Während die kontinuierlich hohe Expression von Genen der GH3-Proteinfamilie, die eine wichtige Funktion in der Auxinkonjugierung einnimmt, zu der Reduktion des IAA-Pools nach dem 24-Stunden Peak (**Ahkami et al., 2013**) beitragen kann, deuten phasenabhängige Expressionsmuster von Genen der Auxinefflux und -influxtranporter auf eine Feinjustierung des Auxintransportapparates hin (**Druege et al., 2014**). Insbesondere die beobachtete phasenspezifische Regulation von Aux/IAA Proteinen als Auxinrepressoren und der Auxin-Response-Faktoren (ARFs) als Auxinsignalgeber wurden als wichtige Steuerkomponenten der auxin-regulierten AW-Bildung diskutiert. Aus den Daten wurde ein Konzept abgeleitet, in dem die frühe PAT-abhängige Auxinakkumulation in Folge der Abtrennung von dem basipetalen Abtransport als Initialereignis betrachtet wird, das eine nachfolgende Autoregulation des Auxintransportes und der Auxinsignaltansduktion einleitet, die einerseits der Auxinakkumulation entgegenwirkt und andererseits die AW-Bildung einleitet (**Druege et al., 2014**).

In der Diskussion wurde unter Berücksichtigung der weiteren jüngeren Literatur das Auxin- und Ethylen-Konzept um die Prozesse der Auxinkanalisierung und -maximierung als wesentliche treibende Prozesse der zellulären Differenzierung, um die GRAS-, AP2/ERF- und WOX-Transkriptionsfaktoren als wichtige Mittler des Auxinsignals und um die Regulation des Zellzyklus und die Remodellierung der Microtubuli und der Zellwand erweitert sowie um die Jasmonsäure als weiteres wund-responsives Signal ergänzt (Druege et al., 2016). Darüber hinaus wurden basierend auf den 14 oben dargestellten eigenen Untersuchungen 12 Konzepte zu wesentlichen limitierenden Faktoren und Prozessen abgeleitet, welche die AW-Bildung und Blattseneszenz in Kopfstecklingen im Rahmen der Jungpflanzenproduktionskette limitieren, und in ein Gesamtmodell integriert. Die räumliche Zuordnung der Schlüsselprozesse zu den Geweben bzw. Zellen in der Sprossbasis und die funktionale Analyse der identifizierten Kandidatengene mittels vorhandener Transposon-Mutanten und/oder genetischer Transformation von *P. hybrida* werden als wichtige zukünftige Schritte angesehen, das Verständnis der Steuerung der AW-Bildung in Stecklingen entscheidend zu erweitern. Die kürzlich publizierten komplett sequenzierten und annotierten Genome von zwei Urspungsarten von *P. hybrida* bieten die Möglichkeit, über Nachkommenschaftsanalysen die AW-Bildung kontrollierenden Abschnitte des Genoms zu identifizieren und die funktionale Analyse von Kandidatengenen auf diese Genabschnitte zu fokussieren. Um die Wirkung von Umweltfaktoren weiter aufzuklären, sollte der Ansatz, weitere Teile des Stecklings hinsichtlich ihrer physiologischen Funktion und Umweltreaktion zu charakterisieren und in ein mechanistisches Modell des umwelt-responsiven Gesamtsystems “Steckling” zu integrieren, weiter verfolgt werden. Letztendlich ist es notwendig, die am Modellsystem Petunie identifizierten Schlüsselfaktoren und -prozesse hinsichtlich ihrer Gültigkeit für verschiedene Petuniensorten und verschiedene Pflanzenarten zu überprüfen.

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