Molecular physiology of chilling tolerance of *Petunia hybrida*

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

Diplom-Biologe Univ. Martin Andreas Bauerfeind

geboren am 02.12.1983 in Kulmbach

2016

Referentin: Prof. Dr. Traud Winkelmann

Korreferent: Prof. Dr. Thomas Debener

Tag der Promotion: 12.08.2016

Meinen Eltern

Die Wissenschaft hat das mit der Kunst gemein, daß ihr das Alltäglichste völlig neu und anziehend, ja wie durch die Macht einer Verzauberung als eben geboren und jetzt zum ersten Male erlebt erscheint. Das Leben ist wert, gelebt zu werden, sagt die Kunst, die schönste Verführerin; das Leben ist wert, erkannt zu werden, sagt die Wissenschaft.

Friedrich Wilhelm Nietzsche
Homer und die klassische Philologie. Ein Vortrag (1869)

.

Abstract

The production of petunia (*Petunia hybrida*) during winter consumes huge amounts of heating energy. Thus, the cultivation of chilling-tolerant petunia cultivars at reduced temperatures would enable a more sustainable production by reducing both, energy costs and greenhouse gas emissions. Since the molecular physiological chilling response of petunia in general and the responsible reaction patterns for chilling tolerance in particular are widely unknown, research is crucial. Therefore, the following categories were investigated: the expression of genes which build the basis for stress responses; phytohormones that act as stress tolerance modulating players; and the carbohydrate metabolism that can be influenced by low temperatures in its essential functions of translocation of carbohydrates for supply of energy and building material for new plant matter.

Therefore, the aim of this thesis was to answer the following questions:

1.) Can chilling-sensitive/tolerant cultivars be identified?
2.) How can the chilling response of a chilling-sensitive cultivar on the levels of gene expression, phytohormone status and carbohydrate metabolism be characterized?
3.) Which reaction patterns and which candidate genes can be related to chilling tolerance?

Under chilling (12 °C vs. 16 °C), the growth of ten petunia cultivars was evaluated. Chilling reduced biomass production, elongation growth and leaf development. Branching of lateral shoots was less affected by chilling, but high rates of lateral shoot development seemed to support chilling tolerance. A repeatable parameter to evaluate chilling tolerance was dry weight production. Thus, indices based on this parameter were introduced to compare the individual chilling responses between cultivars as well as general cultivar-specific growth potentials of the investigated cultivars. 'Sweet Sunshine Williams' (SW) was selected as chilling-sensitive cultivar and 'Ultra Blue' (UB) as tolerant one.

A response model for the chilling-sensitive cultivar SW is proposed. Young differentiated leaves were analyzed as source organs, the apex as sink organ, and the upper stem as transport unit. Chilling responses were detected at the levels of carbohydrates, phytohormones, and gene expression. Acute exposure to chilling deranged the plants' homeostasis, followed by a transient phase of recovery marked by a trend towards values of control conditions. As long-term acclimation to chilling, new equilibria seemed to be established.

To characterize chilling tolerance, the chilling response of the chilling-tolerant UB was compared with the chilling-sensitive SW. Chilling tolerance correlated with a generally better carbon translocation to and a higher abundance and a better utilization in the apical tissue. These findings were supported by general higher sucrose levels as well as higher invertase activities in the apex of UB. Abscisic acid (ABA) might play a key role for chilling tolerance. Especially in the apex, UB displayed generally higher ABA concentrations at both temperatures. The results of an experimental ABA-treatment of SW and an NDGA(ABA-biosynthesis inhibitor)-treatment of UB supported the hypothesis of a protective role. It is discussed how far transcription analyses support these findings and provide indications for candidate genes for chilling tolerance. In contrast to the chilling-sensitive cultivar SW, the tolerant cultivar UB seemed to follow a growth priority rather than a defense strategy at mild chilling stress.

This work generates a better understanding of the molecular mechanisms of chilling tolerance in *P. hybrida* and suggests new candidate genes for chilling tolerance. The insights presented can be very helpful for future evaluation as well as for the selection and breeding of chilling-tolerant cultivars.

Key words: chilling tolerance, *Petunia hybrida*, stress

Zusammenfassung

Die Produktion von Petunien (*Petunia hybrida*) im Winter verbraucht große Mengen an Heizenergie. Hier könnte der Anbau kühletoleranter Sorten bei reduzierten Temperaturen eine nachhaltigere Produktion durch reduzierte Energiekosten und Treibhausgasemissionen ermöglichen. Da die molekularphysiologische Kühlereaktion von Petunie im Allgemeinen und die für die Kühletoleranz relevanten Reaktionsmuster im Besonderen weitgehend unbekannt sind, besteht Forschungsbedarf. Die folgenden Kategorien wurden untersucht: Die Genexpression, die die Grundlage der Stressreaktion bildet; Phytohormone, die die Stresstoleranz beeinflussen; und der Kohlenhydrat-metabolismus, der bei Kühle essentiell in seiner Funktion der Kohlenstofftranslokation zur Versorgung mit Energie und Baumaterial für neues Pflanzengewebe beeinträchtigt sein kann.

Daher war es Ziel dieser Arbeit die folgenden Fragen zu beantworten:

1.) Können kühlempfindliche/tolerante Sorten identifiziert werden?
2.) Wie kann die Kühlereaktion einer kühlempfindlichen Sorte auf Ebene der Genexpression, des Kohlenhydratmetabolismus und des Phytohormonstatus charakterisiert werden?
3.) Welche Reaktionsmechanismen und Kandidatengene sind für die Kühletoleranz relevant?

Das Wachstum von zehn Petuniensorten unter Kühle (12 °C vs. 16 °C) wurde evaluiert. Die Kühle reduzierte Biomasseproduktion, Streckungswachstum und Blattbildung. Das Verzweigungswachstum war kaum von der Kühle betroffen, generell höhere Verzweigungsraten schienen aber die Kühle-toleranz zu unterstützen. Ein reproduzierbarer Parameter zur Bestimmung der Kühletoleranz war die Trockenmasseproduktion. Darauf basierende Indizes wurden eingeführt, um sortenspezifische Wachstumspotenziale, sowie individuelle Kühlereaktionen verschiedener Sorten zu vergleichen. ‚SweetSunshine Williams' (SW) wurde als kühleempfindliche Sorte und ‚Ultra Blue' (UB) als tolerante gewählt.

Ein Modell für die Kühlereaktion der kühlesensitiven SW wird vorgeschlagen. Junge differenzierte Blätter wurden als „Source"-Organ untersucht, der Apex als „Sink"-Organ und die obere Sprossachse als Transporteinheit. Kühlreaktionen waren nachweisbar auf Ebene der Kohlenhydrate, der Phytohormone und der Genexpression. Die akute Kühleexposition destabilisierte die Homöostase der Pflanze, gefolgt von einer vorübergehenden Erholungsphase mit einem Trend zu den Werten unter Kontrollbedingungen. Als langfristige Kühleakklimatisierung schienen sich neue Gleichgewichte einzustellen etablieren.

Zur Charakterisierung der Kühletoleranz, wurden die Kühlereaktionen der kühletoleranten Sorte UB und der ~sensitiven SW verglichen. Die Toleranz korrelierte mit einer generell besseren Kohlenstoff-translokation sowie höheren Überschüssen und besserer Nutzung im Apex. Diese Ergebnisse wurden durch generell höhere Saccharosegehalte und höhere Invertaseaktivitäten im Apex von UB gestützt. Abscisinsäure (ABA) könnte eine Schlüsselrolle für die Kühletoleranz spielen. Vor allem im Apex zeigte UB deutlich höhere ABA-Konzentrationen bei beiden Temperaturen. Die Ergebnisse einer experimentellen ABA-Behandlung von SW und NDGA(ABA-Biosyntheseinhibitor)-Behandlung von UB unterstützten die Hypothese einer protektiven Rolle von ABA. Es wird diskutiert, in wie weit die Transkriptionsanalysen diese Erkenntnisse unterstützen und Hinweise auf Kandidatengene für die Kühletoleranz liefern. Im Gegensatz zu der sensiblen Sorte SW schien die tolerante Sorte UB bei mildem Kühlestress einer Wachstumspriorität, anstatt einer Verteidigungsstrategie zu folgen.

Diese Arbeit schafft ein besseres Verständnis der molekularen Mechanismen der Kühletoleranz in *P. hybrida* und schlägt neue Kandidatengene für die Kühletoleranz vor. Die beschriebenen Erkenntnisse können der zukünftigen Evaluation, sowie Züchtung und Selektion kühletoleranter Sorten dienen.

Schlagworte: Kühletoleranz, *Petunia hybrida*, Stress

Contents

Abbreviations

ABA	abscisic acid
ACP	acyl carrier protein
ASR	abscisic stress ripening protein
ATPase	adenylpyrophosphatase
Aux	auxin
bHLH	basic helix-loop helix
bZIP	basic leucine zippers
CBF/DREB1	C-REPEAT BINDING FACTORS/DEHYDRATION-RESPONSIVE-ELEMENT-BINDING PROTEIN
CC	climate chamber experiment
C category	genes with chilling-independent cultivar-specific hybridization signals
CC category	genes with chilling-dependent cultivar-specific hybridization signals
cDNA	complementary DNA
Com Fam EP	Compact Famous Electric Purple
Com Fam HP	Compact Famous Hot Pink
CPI	chilling performance index
CPsHSP	chloroplast-localized small heat shock protein
CTI	chilling tolerance index
cv(s)	cultivar(s)
cwINV	cell wall invertase
cytINV	cytoplasmic invertase
DELLA	DELLA family of proteins, named after the conserved Asp-Glu-Leu-Leu-Ala N-terminal motif.
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
DoT	Days after differentiation of temperature
DREB	DEHYDRATION-RESPONSIVE-ELEMENT-BINDING PROTEIN
DW	dry weight
ERF	ethylene responsive factor
EST	expressed sequence tag
Fam DB	Famous Dark Blue
Fam F	Famous Firestorm
Fam LB	Famous Light Blue
Fam LDV	Famous Lilac Dark Vein
Fru	fructose
FW	fresh weight
GA	gibberellin
GC-MS/MS	gas chromatography-tandem mass spectrometry
Glc	glucose
GH1-3	greenhouse experiment 1-3
GI	growth index
GID1	GIBBERELLIN-INSENSITIVE DWARF 1
GID2	GIBBERELLIN-INSENSITIVE DWARF 2
H^+-ATPases	proton adenylpyrophosphatase
HSP	heat shock protein
IAA	indole-3-acetic-acid
ICE	INDUCER OF CBF EXPRESSION

JA	jasmonic acid
jmjC	Jumonji domain containing
MADS	minichromosome maintenance agamous deficiens
MYB	myeloblastosis
NDGA	nordihydroguaiaretic acid
n.s.	not significant
PEP	phosphoenolpyruvate
P. hybrida	*Petunia hybrida*
pfp	probability of false prediction
PIFI	postillumination chlorophyll fluorescence increase
PPFD	photosynthetically active photon flux density
PS	photosynthase
RNA	ribonucleic acid
RNAi	RNA interference
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SCF	Skp1p-Cdc53p-F-box protein
SEQ_ID	sequence identifier
Suc	sucrose
SAUR	*SMALL AUXIN UP RNAs*
SW	SweetSunshine Williams
SwSu Bur	SweetSunshine Burgundy
SwSu WE	SweetSunshine White Evolution
SwSu Will	SweetSunshine Williams
TF	transcription factor
UB	Ultra Blue
vacINV	vacuolar invertase
WeGa	Wertschöpfungskette Gartenbau (Horticultural Research Network)
Williams	SweetSunshine Williams
WRKY	named after WRKYGQK peptide sequence domain of DNA binding element

1 General introduction

The impending ecological crisis due to manmade climate change, increasing energy prices due to the shortage of energy and growing ecological awareness of the customers require the best possible sustainability of plant production. To comply with all these demands and to produce still profitably, the greenhouse production of ornamentals in Central Europe needs an efficient usage of all available resources. The greenhouse production of petunia in Germany from the middle of February until early April requires huge amounts of heating energy, especially during the first weeks. Heating of greenhouses accounts for about 90 % of the total energy consumption of German horticultural production (Gabot.de, 2012). Thus, especially for thermophilic ornamentals like petunia, which are produced during winter months and early spring, heating entails a big potential for savings of energy costs. To realize the full saving potential, various approaches can be applied to reduce energy costs in the greenhouse production. Improvements in the thermal insulation of greenhouses as well as optimization of temperature management are commonly used approaches to save heating energy. Another important option of saving energy is a production at lower temperatures. Elings et al. (2005) stated energy savings of 16 % for greenhouse production of tomato, realized by a 2 K reduction of the mean cultivation temperature. However, to avoid longer production times due to chilling-induced growth depression, the use of chilling-tolerant cultivars or species is crucial. In this context, a 'chilling-tolerant' cultivar is a cultivar that displays a significantly smaller growth depression than the average of other cultivars of the same species at sub-optimal production temperatures compared to commonly used production temperatures. Best for production, of course, would be a cultivar that shows a similar growth performance at sub-optimal temperature, resulting in a similar development time for the production of marketable products of comparable quality.

1.1 Petunia: An ornamental of economic importance and a model plant for research in ornamental crops

Petunia is one of Germany's favorite ornamentals. With a market volume of more than 110 million Euros, petunia belonged to the German top five balcony and bedding plants in 2012 (BMEL, 2014). Likewise, it is popular in other European countries and North America (Kelly et al., 2007).

Beyond that, petunias already have a long history as cultivated bedding plants. The *Petunia* species belong to the family of *Solanaceae* and originate from South America. The modern *Petunia x hybrida* has been cultivated since the early 19th century. It was created by the hybridization of two *Petunia* species, the purple flowering *Petunia axillaris* and the white flowering *Petunia inflata*. Not only because of its genetic similarity to other scientifically and commercially important *Solanaceae* crop species like *Nicotiana tabacum* (tobacco), *Solanum lycopersicum* (tomato), and *Solanum tuberosum*

(potato), petunia is one of the best-investigated model plants in the research of ornamental crops (reviewed in Gerats and Vandenbussche, 2005). Furthermore, due to its more differentiated habitus and the general diversity in forms, compared to the rosette-like growth of *Arabidopsis*, as well as ecological niches, petunia species can be used to answer more complex questions than *Arabidopsis thaliana*. Petunias can easily be asexually propagated and are simple to cultivate. Some petunia lines as the double haploid *Petunia hybrida* cultivar `Mitchell´ can be easily transformed and large numbers of mutants are available. Furthermore, a broad variety of biochemical analysis methods have been well established for physiological and genetic analyses in petunia (reviewed in Gerats and Vandenbussche, 2005). Likewise, genetic engineering in petunia has a long history. Thus, the first field test with transgenic plants in Germany more than 25 years ago was conducted with transgenic petunia carrying a maize gene encoding for a dihydroflavonol reductase (Meyer et al., 1992). Today, the whole genome of both parental species of *Petunia x hybrida*, *Petunia axillaris* and of *Petunia inflata* is sequenced and currently annotated (unpublished, online article from Vandenbussche (2015)). Furthermore, a petunia specific microarray is available for gene expression analyses (Breuillin et al., 2010; Ahkami et al., 2014).

1.2 Chilling: an abiotic stressor

Plant growth and productivity can be adversely affected by a huge number of biotic and abiotic stressors delivered by the environment. The resulting plant condition in response to these exogenous stressors is stress. On the one hand, the plant growth and development can be severely reduced, when a plant needs to engage its resources to fight against pathogens such as viruses, bacteria, fungi, and herbivores. On the other hand, plant growth can also be negatively influenced by a great number of abiotic environmental stress factors such as oxidative stress, heavy metal toxicity, flooding or drought, salt surplus or nutrient deficiencies as well as low temperature or heat. Hereby, temperature is an important stress factor that influences development, growth performance and habitus of a plant. For every plant species a specific temperature range exists, which can be defined as optimal for plant development and growth. As soon as the surrounding temperatures decrease below this specific optimal temperature range, the effect of cold temperatures on the plant metabolism can be considered as abiotic stimuli that impose stress to the plant. However, the literature distinguishes between cold reactions at very low temperatures and freezing reactions below 0 °C on the one hand, and chilling reactions at still moderate, but sub-optimal temperatures on the other hand. While the cold and freezing reactions are pretty well investigated, much less literature is available for chilling reactions. Especially in research of ornamentals like petunia, the understanding of chilling reactions on a molecular physiological level is still lacking. Nevertheless, the literature concerning chilling reactions has proposed at least three different definitions of chilling temperature. Hogewoning and Harbinson

(2007) define beginning chilling stress when the first physiological reactions can be related to a decreased temperature. Other definitions are linked to a visible growth depression (Allen and Ort, 2001) or even to the experience of visible cold damage or decaying plant parts (Nagarajan and Nagarajan, 2010). In the context of this thesis, the term 'mild chilling' refers to a slightly reduced temperature range compared to commonly used production temperatures at which a growth depression can be realized without any visible cold damage, so that the plant quality is still preserved.

Plants often react to cold with obvious changes of their phenotype like a reduced growth and development (**Figure 1**) depending on their state of development and their genotype, but also because of environmental factors such as the degree and the duration of temperature reduction. Thus, at the same level of temperature reduction, different species but also different subspecies or cultivars within the same species can expose distinct reactions to this stress factor (Walworth and Warner, 2009; Warner, 2010). These changes derive from very complex modifications, which affect the expression of genes as well as the whole metabolism. In *Arabidopsis*, the reactions to slightly reduced temperatures do not seem to differ substantially from the kind of reaction to cold temperatures. Solely the extent and the severity of the reactions increased continually with decreasing temperatures (for an observed temperature range from 20 °C down to 8 °C) (Usadel et al., 2008). At 10 °C - 12 °C, especially thermophilic plants already show a disturbance of the carbohydrate metabolism (Hällgren and Öquist, 1990). In some thermophilic species, already at moderately reduced temperatures, photosynthesis can be inhibited by feedback mechanisms (photoinhibition) as a consequence of the changes regarding the carbohydrate metabolism and thereby increased carbohydrate levels in source tissues (Bagnall et al., 1988). Additionally, also the phytohormone metabolism is involved in the reaction to chilling. However, all efforts a plant has to take to cope with the stress imposed by a stressor need energy at the expense of growth or at least of reserves. The next chapters will focus on these chilling reactions in detail.

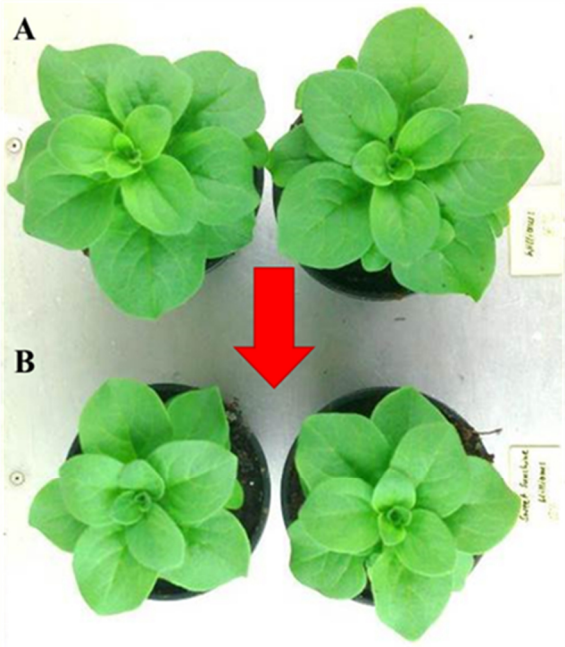


Figure 1 | Growth depression after exposure to chilling.

Chilling-sensitive cultivar 'SeetSunshine Williams' after three weeks of differentiated temperature treatment with a control temperature of 16°C (A) and a sub-optimal temperature of 12°C (B).

Photo: Martin Bauerfeind

1.2.1 Changes in gene expression in response to chilling

The modification of the gene expression plays a crucial role in the plant's reaction to chilling, which enables the plant to cope with stress stimuli. Thus, the growth depression and physiological changes as response to cold exposure are the result of a complex disturbance of a variety of genetic and molecular physiological processes. Carbohydrate metabolism and transport are affected as well as photosynthesis or the homeostasis of phytohormones (1.2.2, 1.2.3). These changes might partly be related to direct temperature-dependent physical effects, like chilling-dependent lower enzyme activities when temperatures are far below the temperature for optimal enzyme activity. Furthermore, activation or inhibition mechanisms of various enzymes can be changed under reduced temperatures (Sasaki et al., 2001). However, reduced temperatures affect gene expression and enhance or repress the transcription of whole groups of genes. Thus, several cold-responsive pathways are known. How effective such pathways are activated seems to be an important determinant for cold tolerance.

The plant´s sensors for chilling stress are not fully identified yet (Chinnusamy et al., 2010). However, through detecting low temperature-induced changes in membrane fluidity, metabolite concentrations and/or nucleic acid and protein conformation plants can sense cold stress. Pharmacological rigidification (reduction of membrane fluidity) of plasma membranes in alfalfa and *Brassica napus* induced *cold-responsive* genes (*COR*) (Chinnusamy et al., 2010). Membrane rigidification-induced activation of Ca^{2+} channels increases Ca^{2+} influx and Ca^{2+}-dependent phosphorylation, which are involved in cold stress signal transduction (Viswanathan and Zhu, 2002). Ca^{2+}-mediated cold signaling can be also influenced by secondary signals like reactive oxygen species (ROS) or abscisic acid signaling. The accumulation of ROS seems to have a strong effect on the cold regulation of gene expression, but also enzymes like kinases, phospholipases and phosphatases can be involved in the signaling cascades (reviewed in Chinnusamy et al., 2007). One of the best investigated cold-response pathways, that plays a key role for cold acclimation in *Arabidopsis* and others, is the C-repeat binding factor (CBF) cold-response pathway (reviewied in Thomashow, 1999). These CBF proteins can activate the expression of *COR* genes by binding to cis-elements in their promotors and thereby improving cold/freezing tolerance (Thomashow, 2001). Exemplary in *Arabidopsis*, the transcript level of CBF genes accumulated within 15 min after exposure to cold (Gilmour et al., 1998). The transcription of CBF genes at cold temperature on the other side is regulated by the upstream transcription factor ICE1 (inducer of CBF expression 1) (Chinnusamy et al., 2003). The ICE1 cascade seems to be crucial for the expressional regulation of chilling tolerance, at least in *Arabidopsis* (reviewed in Chinnusamy et al., 2007). However, this is not the only cold-responsive pathway. Also several non-CBF transcription factors exist that induce the expression of *COR* genes in *Arabidopsis* (reviewed in Chinnusamy et al., 2007). Furthermore, post-transcriptional regulation of genes might play important roles in the cold response. Thus, Zhou et al. (2008) supposed that cold-inducible

microRNAs (non-coding ~ 21-nucleotide long RNAs) may affect many signaling pathways, such as auxin pathways.

The cold-induced changes in gene expression affect a broad range of the plant's metabolism and protection mechanisms. For instance, the expression of photosynthesis related genes can be repressed (Janská et al., 2010). Ruelland and Collin (2016) found an accumulation of proteins with a protective chaperone-like function like LEA proteins (late embryogenesis abundant), a kind of dehydrins protecting plants against cellular damage by stabilizing membranes. Further, the synthesis of heat shock proteins that display chaperone-like functions can be increased by cold (Taiz and Zeiger, 2008). For example, the constitutive expression of a gene for the tomato chloroplast-localized small molecular heat-shock protein (CPsHSP) lead to a weaker damage to photosynthesis, resulting in a higher net photosynthetic rate (Wang et al., 2005). Additionally, pathogenesis-related proteins like ß-glucanases, chitinase, lipid-transfer proteins and thaumatin-like proteins can be affected by cold and be more synthesized (Janská et al., 2010).

Current studies on petunia suggest the existence of several different signaling systems, whose interplay is substantial for the cold stress response (Li et al., 2015). The above-mentioned CBF cold-responsive pathway also seems to be among these pathways. Walworth et al. (2014) increased the freezing tolerance of *P. hybrida* with the ectopic expression of *AtCBF3*. By that, the authors found evidence that a functional CBF cold-responsive pathway also exists in petunia.

The abilities of non-targeted transcriptomic approaches have been increased enormously during the last decade, even for species like petunia. Therefore, Breuillin et al. (2010) have applied EST sequences from cDNA libraries derived from *P. hybrida* and *P. axillaris* control roots, mycorrhizal roots, and phoshate-treated roots to create a petunia specific microarray for gene expression analysis. Our research group at the Leibniz Institute of Vegetable and Ornamental Crops (IGZ) actively joined the development of this microarray. It provides 24,816 non-redundant unique sequences (Breuillin et al., 2010; Ahkami et al., 2014).

1.2.2 Relationship between the plant carbohydrate metabolism and the chilling response of plants

Carbohydrates in plants are the products of carbon dioxide fixation by photosynthesis. They play an essential role for the storage and translocation of energy, and as building material of new plant matter. Plant tissues displaying a net carbohydrate synthesis such as mature green leaves are categorized as carbohydrate source tissues. Plant organs with a net utilization of carbohydrates such as meristematic tissues, fruits, flowers or roots are categorized as carbohydrate sink tissues. Reduced temperatures especially influence the carbohydrate metabolism. Besides carbohydrate accumulation in the source

leaves, changed ratios of carbohydrate synthesis, transport and consumption are also the consequences of reduced temperatures (Druege and Kadner, 2008). Especially at very low temperatures approaching the freezing point, these increases of osmolyte concentrations may protect the cells from freezing (Mahajan and Tuteja, 2005). However, carbohydrates already accumulate at slightly reduced temperatures, even when cryoprotection is not vital for survival at those mild chilling temperatures. For example, in the newest mature source leaves of tomato, increased levels of sugar and starch were already observed as a response to a mild chilling (day/night temperature: 16/14 °C vs. 25/20 °C) (Venema et al., 1999). Nevertheless, one reason for the increased sugar levels, especially of sucrose, might be a change in enzyme activities that are involved in the carbohydrate metabolism. Thus, in *Arabidopsis*, the activities of the two sucrose synthesis enzymes fructose-1,6-bisphosphatase and sucrose-phosphate synthase were slightly increased in leaves after exposure to 5 °C and strongly increased in young leaves, which were fully developed at 5 °C (Strand et al., 1997). Guy et al. (1992) found higher sucrose-phosphate synthase activities in spinach source leaves under exposure to chilling. Since sucrose functions as a storage of fructose and glucose under exposure to abiotic stress, which can be fast and easily mobilized as an energy source that is translocated to utilization sinks (Guy et al., 1992), the accumulation under exposure to chilling seems to help the plants to maintain their metabolism. Thus, Sin'kevich et al. (2008) showed that cold resistant potato plants could adapt better to a temperature reduction, when they accumulated low-molecular carbohydrates by the activation of acid invertases.

However, on the one hand, the increased carbohydrate levels in the source tissues might indirectly negatively affect the plant growth as long as the increase in the source leaves results from a reduced carbohydrate translocation to the utilization sinks. On the other hand, beside their importance for the maintenance of energy supply, sucrose and hexoses also play an important role as signal molecules, which are generally involved in the source-sink-regulation, and especially under exposure to stress-related stimuli like cold (reviewed in Roitsch, 1999). Thus, photosynthesis can be inhibited in the photosynthetic active tissues by end-product accumulation of sugars (Goldschmidt and Huber, 1992). This temperature-dependent feedback inhibition of photosynthesis arises in thermophilic species like peanut already at moderate temperatures like 15 °C compared to an optimum of 30 °C (Bagnall et al., 1988). Generally, cold-tolerant plants seem to be more flexible in various photosynthetic parameters during cold acclimation (Yamori et al., 2010) and cold tolerance also appears to be related to a better temperature homeostasis of leaf respiration and photosynthesis (Yamori et al., 2009).

1.2.3 Relationship between phytohormones and the chilling response of plants

Phytohormones control the growth and development of plants and mediate nutrient allocation as well source/sink transitions. Beyond that, they are the key players adjusting the reactions to abiotic and

biotic stimuli, and enable plants to adapt to a changing environment (Peleg and Blumwald, 2011). Several phytohormones are also involved in the chilling response. Cytokinins stimulate cytokinesis in plant roots and shoots. Under exposure to endogenous or exogenous increased cytokinin levels at 4 °C *Arabidopsis* showed higher relative growth rates by increasing total cell numbers (Xia et al., 2009). Salicylic acid is a phytohormone that regulates plant growth and development, and is also involved in the plant defense against pathogens. Likewise, it plays a role in the response to abiotic stresses. Thus, salicylic acid accumulates at low temperatures. Scott et al. (2004) showed that at 5 °C mutants, in which this accumulation at low temperatures was inhibited, grew faster than wild-type *Arabidopsis*. On the other hand salicylic acid treatment was shown to protect tomato from chilling injuries (Ding et al., 2002). The gibberellin class of growth hormones regulates developmental processes like stem elongation, germination, dormancy, leaf senescence and others, and is also involved in the response to abiotic stress, including cold. The cold-responsive CBF pathway (see 1.2.1) mediates a reduction in bioactive gibberellins, which promotes an accumulation of DELLA proteins. An accumulation of these DELLA proteins results in a growth restriction, but enhances freezing tolerance (Achard et al., 2006). Ethylene is a gaseous phytohormone, which controls numerous cellular and developmental processes, and has an important role during the abiotic and biotic stress response (reviewed in Abeles et al., 1992 and in Gallie, 2015). Shi et al. (2012) found that ethylene negatively regulates cold signaling through direct transcriptional control of cold-regulated CBFs. Therefore, ethylene seems to reduce cold/freezing tolerance.

Abscisic acid (ABA) was first described as a leaf abscission- and seed dormancy-promoting phytohormone. Today it is known to play an important role as an endogenous messenger in the plant's response to biotic and abiotic stress factors. Especially high salinity and drought cause strong increases of ABA levels (reviewed in Raghavendra et al., 2010). Nevertheless, ABA also seems to be critical for the chilling tolerance, since an adequate regulation of endogenous ABA levels is supposed to be critical for maintaining cold tolerance, at least in rice (Mega et al., 2015). Mild chilling tolerance of tomato can be related to the ABA biosynthesis, even if in this case no accumulation of ABA seems to be necessary (Ntatsi et al., 2013). However, application of exogenous ABA induces freezing tolerance in wheat and chilling-sensitive rice seedlings (Veisz et al., 1996; Shinkawa et al., 2013). Transgenic rice lines overexpressing the enzyme ABA 8′-hydroxylase (OsABA8ox1), which is involved in ABA catabolism, display reduced ABA levels. While even reduced ABA levels still seemed to support chilling tolerance, excessively low levels caused reduced cold and drought tolerance (Mega et al., 2015). Nevertheless, ABA seems to regulate many genes, which are positively associated with freezing tolerance. Likewise, Gusta et al. (2005) suggested that ABA-dependent pathways, beside ABA-independent ones, are part of the cold-response. Thus, the CBF pathway is also induced by exogenous ABA application (Knight et al., 2004).

Jasmonic acid (JA) is an important regulator of the wound response in many plants, including petunia, and also involved in the regulation of primary metabolic functions such as sucrose and starch accumulation (Ahkami et al., 2009). While wounding is often caused by biotic stressors like herbivores, JA also plays a role in the chilling response. Thus, exogenous application of JA enhances cold tolerance (Ding et al., 2002; Fung et al., 2004). On the one hand, the balance of JA and IAA homeostasis and signaling seem to be important for development in general and in stress response in particular (Du et al., 2013). On the other hand, JA also activates freezing tolerance-enhancing pathways. Jasmonates improve the freezing tolerance in *Arabidopsis* by acting as a critical upstream signal of the CBF/DREB1 pathway, which is a key player of chilling tolerance in *Arabidopsis* (Hu et al., 2013).

Auxin is a key growth hormone that regulates all stages of the plant development from embryogenesis to senescence. Furthermore, auxin plays an essential role in the existing hormonal crosstalk, which also affects several developmental stages (reviewed in Rahman, 2013). The metabolism and transport of auxins are affected by sub-optimal temperatures. Thus, in carnation cuttings, the transport of auxins is impaired by sub-optimal temperatures (Garrido et al., 2002). However, the cold treatment gradually enhanced the auxin response in tulip bulbs over the course of 12 weeks (Rietveld et al., 2000). Conversely, in *Arabidopsis*, auxin-inducible genes are down-regulated in response to cold (Lee et al., 2005). The authors suppose that the cold-induced disturbance of auxin homeostasis, signaling and transport might be involved in the cold-induced downregulation of auxin-inducible genes. In consequence, the growth depression, caused by cold, might be partially triggered by the downregulation of auxin transport and auxin-responsive genes (Lee et al., 2005).

1.2.4 Impact of chilling on the growth of petunia

On the phenotypical level, some research has already been done regarding the temperature needs as well as the response to sub-optimal temperatures of petunia. For the cultivar `Snow Cloud´ Kaczperski et al. (1991) described a decrease in plant height and average internode length accompanied by a reduced length of individual shoots but a higher number of lateral shoots for a progressive temperature reduction from 30 °C to 10 °C. Since petunia is famous for its plentiful blossom, several studies focused on the production time to flower and the quality of the flowering plants. For plant producing companies, both are very important issues for producing good, marketable quality. Thus, Warner (2010) analyzed the time to flower of four *Petunia* species, including *P. hybrida*. In a temperature range from 14 °C to 26 °C, for all four species the days to flower decreased with increasing temperature and the plants had formed fewer nodes under the first flower. At 14 °C, the time to flower required by *P. hybrida* cultivar ‘Mitchell’ was about 20 days longer than at 17 °C, whereas the number of flower buds remained relatively stable (Warner, 2010). In contrast to

'Mitchell', the number of flower buds of nine analyzed grandiflora-type *P. hybrida* cultivars declined at increasing temperatures from 14 °C to 26 °C (Warner, unpublished data, cited in Warner [2010]). Other studies on other *P. hybrida* cultivars confirmed the prolonged time period to flower at sub-optimal temperatures (Adams et al., 1998; Adams et al., 1999; Blanchard et al., 2011b). Thus, the optimal temperature for minimizing the time to flower seemed to be 26 °C or more, at least for the four petunia species Warner (2010) investigated. This temperature range for the optimal development was also proven by the findings of earlier studies (Adams, 1999; Kaczperski et al., 1991; Lieth et al., 1991). Nevertheless, since such high temperatures also promote length growth of the shoots and internodes respectively, and reduce the number of flower buds (Warner, 2010; Blanchard et al., 2011b), the optimal development temperatures are not automatically optimal production temperatures. Since temperatures below the optimum of 26 °C cause a more compact growth with higher flower bud numbers, the optimal temperatures are not used in common production. Moreover, Blanchard et al. (2011a) even established models to calculate the impact of the temperature and the photosynthetic daily light integral and their interactions on the development time and quality of selected petunia cultivars, based on parameters like plant height, number of flowers and time to flower. Taking the longer cultivation time at reduced temperatures into account on the one hand and energy savings on the other, Blanchard et al. (2011b) predicted the lowest heating costs for an average temperature between 14 °C and 17 °C when the finish date of petunia is set in the middle of May. However, because the different petunia cultivars showed differences in their general growth performance and in their chilling response, cultivar-specific adaptions would be required for the practical use of such models.

The above-mentioned reactions differ in extent and manifestation of the reaction between existing *Petunia* species as well as between the different *P. hybrida* cultivars. These differences indicate the existence of divergent chilling-tolerant genotypes in *Petunia* species as well as *P. hybrida* cultivars. Thus, bearing in mind to produce at sub-optimal temperatures, not only the vegetative growth performance at sub-optimal temperature should be considered, which takes place during the season with the highest heating demand, but also the time to flower.

The molecular physiological and genetic backgrounds for distinct chilling reactions between different petunia species or even between cultivars, which are genetically very close, are still largely unknown. Therefore, research is needed to elucidate the underlying mechanisms that are relevant for chilling tolerance. Thus, the present work contributes to a better understanding of chilling tolerance in *Petunia hybrida.*

1.3 WeGa cooperation project: Product- und production safety in intensive plant production systems

The present work `Molecular physiology of chilling tolerance of Petunia´ was embedded in the joint project `Chilling Tolerance of Plants´. The background of this joint project was the crucial importance of future reduction of energy consumption of greenhouse ornamental production in Central and Northern Europe. Consumption of energy and resources could be reduced by producing plants, which display a higher tolerance against chilling stress, at reduced temperatures. Apparently, gene pools of thermophilic plant species like petunia conceal reserves that could be used to enhance chilling tolerance through breeding. The objective of the joint project was to explore knowledge regarding the molecular and physiological regulation of chilling tolerance to integrate low temperature concepts in future greenhouse production. Therefore, the aims and collaborators were:

- Screening of cultivars and physiological investigations
  (petunia and impatiens; Dr. S. Amberger-Ochsenbauer, Weihenstephan-Triesdorf University of Applied Sciences, Freising)
- **Physiological and molecular relations of chilling tolerance**
  (petunia and poinsettia; M.A. Bauerfeind and Dr. U. Druege, Leibniz Institute of Vegetable and Ornamental Crops)
- Genetic modifications: testing of genetic engineering methods in petunia
  (petunia; A. Langhans and H. Mibus-Schoppe (now University of Geisenheim), Leibniz Universität Hannover)
- Physiological conditioning: investigation of an alternative way via symbiotic microorganisms such as arbuscular mycorrhiza
  (petunia and poinsettia; Dr. J. Knopp and Dr. H. von Alten, Leibniz Universität Hannover)
- Screening of cultivars
  (petunia and poinsettia; Dr. D. Ludolph, B. ter Hell, Landwirtschaftskammer Niedersachsen, LVG Ahlem)
- Industrial partner in the joint project: Dümmen Jungpflanzen GmbH & Co. KG; Klemm + Sohn GmbH & Co KG, Selecta Klemm; INOQ GmbH
- Subcontracts: TU München, Gewächshauslaborzentrum Dürnast; TU München Lehrstuhl für Phytopathologie

1.4 Objectives of this thesis

The present work investigates the molecular and physiological background of the response and tolerance of petunia to mild chilling stress, which is still unknown. Observations of commercial breeders showed that some *P. hybrida* cultivars display a better growth performance under exposure to sub-optimal temperatures than the average, and other cultivars stay beneath the average. However, the reasons for those distinct chilling responses remained unidentified. Since savings in heating energy costs are only possible, if the production time at sub-optimal temperatures increases only moderately, genotypes that are only marginally affected by sub-optimal temperatures have to be identified. Thus, the present study describes differences in the phenotypical reactions of petunia cultivars, in order to identify chilling-tolerant genotypes. Based on the growth response, chilling-sensitive and chilling-tolerant cultivars were selected for the investigation of their physiological and molecular chilling responses.

In the context of finding the molecular causes for phenotypical differences, first of all gene expression, which is strongly affected by cold (1.2.1), is analyzed in order to examine, whether a small reduction in cultivation temperature is already leading to explicit changes in the regulation of gene expression. Also, the carbohydrate household is strongly affected by cold (1.2.2). Consequently, at first, concentrations of the sugars fructose, glucose and sucrose, which are essential for the utilization and translocation of energy, were analyzed. Secondly, the relevant invertase activities were analyzed. Most development and growth processes in plants are regulated by phytohormones and several of them are involved in the responses to biotic and abiotic stimuli like chilling stress (1.2.3). Because of the time-consuming and cost-intensive complexity of the phytohormone analyses, in the presented thesis only three important phytohormones abscisic acid (ABA), the auxin indole-3-acetic-acid (IAA) and jasmonic acid/jasmononates (JA) were chosen for the analysis of their role in the chilling response.

Based on the results of these investigations, explanations for the differences in chilling tolerance were deduced.

In detail, the objectives of this thesis were:

1.) Establishment of a reliable and easily applicable screening method for the identification of cultivars with chilling-tolerant and chilling-sensitive responses, respectively.
2.) Characterization of the chilling reaction of a chilling-sensitive cultivar on the levels of phenotypical development, carbohydrate metabolism, phytohormone homeostasis and gene expression.
3.) Formulation of a hypothesis for the basis of chilling tolerance in *P. hybrida*, which is based on the distinct reaction patterns of the phenotypical development, the carbohydrate

metabolism, the phytohormone homeostasis and the gene expression of a chilling-sensitive and a chilling-tolerant cultivar and the identification of new candidate genes involved in chilling tolerance.

1.5 Publications and manuscripts emerged from this thesis

In the following, one publication and two manuscripts are presented, which answer in detail the three above-mentioned objectives of this thesis. A final discussion (Chapter 3 Conclusions and Outlook) condenses the results from the three manuscripts to a coherent overview of the elaborated findings.

1.5.1 Determination of tolerance of *Petunia hybrida* cultivars to mild chilling stress

This manuscript approaches the first aim of this thesis. A set of commercially available petunia cultivars was tested for the phenotypical chilling reactions in air-conditioned greenhouse cabins and climate chambers. The chilling response of the parameters dry and fresh weight, elongation, branching and development of new leaves were measured to determine the growth reaction of the aerial plant to the exposure to chilling. Thus, from the observed growth parameters, fresh and dry weight were proven as the most reliable and repeatable parameters. Based on dry weight production indices are introduced to assess the relative growth potential at a given temperature in relation to the best growing cultivar and to estimate the individual chilling reaction of a cultivar. Furthermore, a reduced susceptibility to chilling of high-branching cultivars is discussed.

The manuscript will be submitted the first time in May 2016 to the peer-reviewed journal Scientia Horticulturae.

1.5.2 Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress

This chapter concerns the second aim of this thesis. It gives a detailed overview of the molecular physiological chilling reaction of the chilling-sensitive cultivar ‘SweetSunshine Williams’. The selection of this cultivar was based on the phenotypical growth analyses of the first investigation approach (Chapter 2.1). ‘SweetSunshine Williams’ already displayed a strong chilling reaction under exposure to mild chilling of 4 K temperature reduction (12 °C compared to 16 °C). This chilling reaction was marked by a complex disturbance of plant functional integrity, featured by a derangement of the carbohydrate metabolism and phytohormone homeostasis, and an altered gene expression. In the paper, a three-phasic model is proposed for the chilling reaction of the sensitive

cultivar ‘SweetSunshine Williams’. The model consists of a derangement during the first days after temperature reduction, a recovery phase after one to two weeks and a phase of stabilization after two to three weeks.

The paper was published on July 28th 2015 in the peer-reviewed open access journal ‘Frontiers in Plant Science’ in the specialty section ‘Crop Science and Horticulture’ (Bauerfeind et al., 2015).

1.5.3 Comparative analysis of two contrasting petunia cultivars indicates important functions of carbohydrate utilization and abscisic acid in tolerance to mild chilling stress

This manuscript approaches the third aim of this thesis. It gives a detailed overview of the distinct molecular physiological chilling reactions of the chilling-sensitive cultivar ‘SweetSunshine Williams’ and the chilling-tolerant cultivar ‘Ultra Blue’. The selection of both cultivars was based on the phenotypical growth analyses described in Chapter 2.1. Both cultivars reacted to the mild chilling stress of a 4 Kelvin temperature reduction (12 °C compared to 16 °C) with a retarded growth, while the growth of the chilling-sensitive cultivar was more affected. The disturbance of plant functional integrity was less in extent and shorter in the chilling-tolerant cultivar compared to the chilling-sensitive one. The analyses of metabolic values and the microarray results indicate a better carbon translocation from the source tissues and utilization in the apex of the chilling-tolerant cultivar ‘Ultra Blue’ at both temperatures, which is less inhibited by chilling. In addition, generally higher ABA levels in the apex of ‘Ultra Blue’ also seem to enhance chilling tolerance.

The manuscript discusses the differential chilling reaction patterns of both cultivars, and draws a hypothesis regarding the contribution of chilling reaction patterns to the chilling-tolerance of ‘Ultra Blue’. Furthermore, it suggests new candidate genes for chilling tolerance in petunia such as genes coding for phosphoenolpyruvate carboxylase kinase or the ABA biosynthesis related carotenoid cleavage dioxygenase and others.

The manuscript has been submitted the first time on May 9th 2016 to the peer-reviewed journal BMC Plant Biology and was at the time of submitting the presented thesis in the process of reviewing.

1.6 Contributions of co-authors to the publications

All figures and statistics of the first manuscript (1.5.1) were created by myself. I performed the phenotypical analyses of the greenhouse experiments at the IGZ (Leibniz Institute of Vegetable and Ornamental crops) in Erfurt. The climate chamber experiment was conducted by Annika Langhans at the University of Hannover (Department of Ornamental Crops, Leibniz Universität Hannover) under

the supervision of Prof. Dr. Heiko Mibus-Schoppe (now at the Department of Urban Horticulture and Ornamental Plant Research, Geisenheim University). The manuscript was written by myself and revised by Dr. Uwe Druege and the co-authors.

All data presented in the second paper (1.5.2) were gained from experiments I performed at the IGZ. The array hybridization of RNA samples was performed by OakLabs (OakLabs GmbH, Hennigsdorf, Germany). OakLabs also executed the normalization of the whole set of array data, which I used for the array analyses. The manuscript was written by myself and revised by Dr. Uwe Druege and the co-authors.

The analysis of photosynthetic rates presented in the third manuscript was conducted by Dr. Susanne Amberger-Ochsenbauer and Florian Steinbacher (both Weihenstephan-Triesdorf University of Applied Sciences, Freising). All remaining data (1.5.3) were gained from experiments I performed at the IGZ. All figures were created by myself. The array hybridization of RNA samples was performed by OakLabs (OakLabs GmbH, Hennigsdorf, Germany). OakLabs also executed the normalization of the whole set of array data. I used this normalized data for the analyses. The manuscript was written by myself and revised by Dr. Uwe Druege and the co-authors.

2 Publications and Manuscripts

2.1 Determination of tolerance of *Petunia hybrida* cultivars to mild chilling stress

Determination of tolerance of *Petunia hybrida* cultivars to mild chilling stress

Martin Bauerfeind[1], Annika Langhans[2], Heiko Mibus-Schoppe[2, 3], Uwe Druege[1]*

[1] Department of Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Erfurt, Germany

[2] Institute of Horticultural Production Systems, Leibniz Universität Hannover (LUH), Hannover, Germany

[3] Department of Urban Horticulture and Ornamental Plant Research, Geisenheim University, Germany

*** Correspondence:** Uwe Druege, Department of Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Kuehnhaeuser Str. 101, 99090 Erfurt, Germany, e-mail: druege@erfurt.igzev.de

Abstract: Growth performance of ten *Petunia hybrida* cultivars (cvs) was evaluated in response to mild chilling (low temperature) stress. Dry weight production, shoot elongation, leaf development and development of lateral shoots were analyzed after four and five weeks, respectively, of chilling treatment in the greenhouse and climate chamber (12 °C compared to a control of 16 °C; two K day/night difference). The applied temperatures were proofed to be appropriate for evaluating mild chilling tolerance in petunia. The cultivar-specific vegetative growth of the investigated cvs, measured by dry weight production, shoot elongation and leaf development, was reduced by chilling, while branching of lateral shoots was mostly not affected. Within the pool of ten cvs, high branching cvs appeared to be more tolerant to mild chilling stress. However, relative growth and growth depression under chilling compared to control conditions featured distinct differences in chilling tolerance among certain cvs, which were independent of general growth characteristics. Here, we introduce three specific indices for a proper evaluation of growth performance at control (growth index = GI) or chilling conditions (chilling performance index = CPI), and the growth robustness to chilling (chilling tolerance index = CTI). GI and CPI can be used to compare the growth performance of a particular cv

at control temperature or chilling, respectively, to the growth of the best cv of a certain pool. To estimate the individual chilling reaction of a cv, CTI can be applied as stand-alone index. Finally, we propose two cvs as models for further physiological and molecular investigations.

Key words: *Petunia hybrida*, chilling stress, cold, chilling tolerance, growth index

1 INTRODUCTION

Petunia (*Petunia hybrida*) was among the five most produced bedding and balcony plant species of Germany with a market share of 6 % of a total of nearly 2 billion euros in 2012 (BMEL, 2014). Thermophilic species like petunia that are produced during winter months and early spring need high amounts of energy for heating and artificial lighting. In German horticultural production, heating of greenhouses accounts for up to 90 % of the total energy consumption (Gabot.de, 2012). While high energy prices and the customers' growing ecological consciousness in the face of climate change request the highest possible sustainability of ornamental production, heating carries a remarkable saving potential of both direct costs and greenhouse gases. Therefore, improved materials for the thermal insulation of greenhouses are already commonly used as well as enhanced adjusted temperature management techniques. Moreover, the optimized combination of both will be increasingly used for production in low energy greenhouses in the future (Schuch et al., 2014). However, the obviously easiest way to save heating energy would be to produce at reduced temperatures. Elings et al. (2005) showed that a 2 Kelvin reduction of the mean cultivating temperature of tomato greenhouse production could save 16 % of heating energy. Since cultivation at reduced temperatures would cause a prolonged production time due to chilling-induced growth depression, chilling-tolerant cultivars (cvs) or species, respectively, are needed. Thus, to maintain an efficient sustainable production at reduced temperatures, plants should be only slightly affected in their growth performance or at least display a significantly smaller growth depression compared to the average of other cvs of the same species. Practical experiences indicate that the degree of chilling tolerance varies among existing commercial petunia cvs. Therefore, to identify distinct chilling-tolerant cvs, reliable and easy to apply criteria are needed for the evaluation of growth response to mild chilling. Especially in ornamentals like petunia, the phenotypical growth reactions on the cv-level aiming at mild chilling tolerance have not been adequately investigated. A more detailed knowledge of the impact of mild chilling stress on phenotypical reactions of petunia cvs would provide an indication on how chilling affects growth and allow establishing selection criteria that provide more efficient breeding towards chilling tolerance. This would also enable a more sustainable greenhouse production.

The term ‘chilling’ commonly labels the temperature range between 15 °C and 0 °C (Lucau-Danila et al., 2012), which is below the optimum temperature of thermophilic crops like petunia. However, the temperature optimum for a minimal time to flower seems to be quite higher with temperatures around 25 - 26 °C or more in petunia (Kaczperski et al., 1991; Warner, 2010). The specific effect of chilling on plants depends mainly on three factors, the degree of temperature reduction, the duration of exposure and the plants’ susceptibility to chilling. Thus, chilling reactions can reach from a reduced plant productivity (Allen and Ort, 2001) up to numerous chilling symptoms such as electrolyte leakage, chlorosis, or severe injuries as necrosis until death of tropical thermophilic plants by nonfreezing low temperatures (Mahajan and Tuteja, 2005). Chilling tolerance describes the capability of plants to cope with these chilling temperatures, while the degree of tolerance varies between plants (Lucau-Danila et al., 2012; Sanghera et al., 2011). That means, chilling-tolerant species are less susceptible to chilling temperatures and show less growth retardation or injuries than the average (Pennycooke et al., 2005; Sanghera et al., 2011). However, tolerance to mild chilling evoked by sub-optimal temperatures cannot simply be determined by evaluating damage symptoms on plants because those are often observed only under severe cold stress. Therefore, easy identifiable growth parameters are needed to evaluate the extent of mild chilling stress or chilling tolerance. The term ‘chilling tolerance’ implies that the plant phenotype or growth under the exposure to chilling has to be evaluated compared to the growth under optimum conditions. For this purpose, many publications use the term ‘performance’ to compare the plant development of a particular cv at specific conditions like chilling with other cvs or with growth at control conditions, while they lack in a proper definition of ‘performance’. In contrast, Kelly et al. (2007) who evaluated growth and development of a huge pool of petunia cvs measuring plant height, width, flower diameter, flowering and days to first flower, defined the overall performance as the average of the flower, foliage, disease and arthropod ratings.

Taking into account that particularly the early vegetative growth of petunia occurs during the winter and early spring with a high demand on heating and to avoid interferences with flower development, the present study focused on the chilling response of vegetative growth. Therefore, based on observations of growers on the response of diverse petunia cvs to varying temperature, a pool of 10 cvs was selected to answer the following questions 1) How large is the variability in growth among the cvs, when cultivated at control temperature of 16 °C (day: 17 °C; night 15 °C) and at sub-optimal temperature of 12 °C (13 °C; 11 °C)? 2) How do different structure parameters of the shoot contribute to chilling tolerance? 3) Which parameters are suitable to compare a) the growth of cvs at the sub-optimal temperature and b) the robustness of growth against sub-optimum temperature?

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND CHILLING TREATMENT

Ten commercial *Petunia hybrida* cvs were screened for differences in their phenotypical chilling reaction: Compact Famous Hot Pink (Com Fam HP), Compact Famous Electric Purple (Com Fam EP), Famous Dark Blue (Fam DB), Famous Firestorm (Fam F), Famous Light Blue (Fam LB), Famous Lilac Dark Vein (Fam LDV), SweetSunshine Burgundy (SwSu Bur), SweetSunshine White Evolution (SwSu WE), SweetSunshine Williams (SwSu Will), Ultra Blue (UB). The pre-selection of these cvs was based on experience of producers who rated these cvs as putative chilling-tolerant, moderate or chilling-sensitive.

For chilling treatment, an average chilling day temperature (24 h mean) of 12 °C was compared to a control of 16 °C average (24 h mean), both with a 2 K day/night difference. This control temperature is frequently used in German greenhouse production of petunia. Preliminary experiments showed that this 4 K reduction in temperature caused a delayed development depending on the cv without visible cold injuries. Young plants were produced from cuttings (Bauerfeind et al., 2015). After potting (pot size: 11 cm; Einheitserde Classic Tonsubstrat ED 73, +Fe, coarse, nutritive salt 1.0, without slow-release fertilizer (GEPAC LCD); Patzer GmbH & Co. KG, Sinntal, Germany), the plants were transferred to greenhouse cabins or climate chambers, respectively. For acclimation, rooted cuttings were transferred into the climate chamber or greenhouse cabin and cultivated on control conditions for two weeks. After this pre-cultivation, half of the plants were relocated to another greenhouse cabin or climate chamber, respectively, with identical climate conditions, but exposed to reduced temperatures for chilling treatment. The other half of the plants remained under control conditions (**Table 1**). Based on the results of the first two experiments in climate chamber (CC, at the Leibniz Universität Hannover, LUH) and greenhouse (GH1, at the Leibniz Institute of Ornamental and Vegetable Crops in Erfurt, IGZ), two additional greenhouse experiments (GH2 & 3, both at the IGZ) were conducted with a reduced number of putative chilling-tolerant and chilling-sensitive cvs, respectively. GH2 and GH3 were conducted simultaneously as two independent repetitions with plants originating from two different batches. In the climate chamber, the indicated targeted values were reached. For the greenhouse experiments, actually measured temperatures are presented. Since investigations focused on vegetative growth only, developing flower buds were constantly removed, as soon as visible, to prevent interference with competing sinks.

2.2 GROWTH PERFORMANCE ANALYSIS

Fresh weights of the aerial plant parts were measured at the beginning and the end of chilling treatments. Dry weights were determined after drying at 80 °C for 48 h until constant weights were reached. To calculate increases in weights, initial weights were subtracted from the end weights. For detailed growth evaluation, the elongation of the main shoot as well as the increases in numbers of

newly developed shoots and leaves of the main shoot (marked at the start of temperature treatment) were evaluated over the period of chilling treatment (for growth conditions see **Table 1**).

2.3 DATA PROCESSING AND STATISTICS

Growth depression in response to chilling was calculated by subtracting individual dry weight increases per plant of each cultivar (cv) at 12 °C from the mean dry weight increase of the same cv at control conditions. As another parameter of growth robustness to chilling, ratios of dry weight increases at 12 °C versus 16 °C were calculated by dividing individual values measured under chilling conditions by the mean value of the measurements of the corresponding control of the same cv. To evaluate the influence of mild chilling stress on the general growth rate, the growth at chilling temperature and the growth robustness against chilling, the growth index (GI), the chilling performance index (CPI) and the chilling tolerance index (CTI) were determined with the five selected putative chilling-tolerant and chilling-sensitive cvs. The GI was calculated by dividing the particular dry weight increases of a specific cv at control conditions by the highest dry weight production found among the pool of cvs at control conditions. Likewise, the CPI was calculated for dry weights under chilling treatment. The CTI was calculated by dividing the individual dry weights of each cv under chilling treatment by the dry weight of the same cv at control conditions. For statistical analyses the STATISTICA software package (StatSoft, Inc. [2014]. STATISTICA for Windows [data analysis software system], version 12.0. www.statsoft.com) was used (Bauerfeind et al., 2015). Different characters indicate different significance groups, significant differences between temperature treatments were marked in the respective figures by asterisks (ANOVA, * $P \leq 0.05$), Spearmans rank correlation coefficient was used to calculate correlation coefficients between the different growth parameters and tested at a significance level of ($P \leq 0.05$).

3 RESULTS

Evaluation of the chilling stress on dry weights

The below-described experiments were conducted in two different research facilities. The climate chamber experiment (CC) was performed at the Institute of Horticultural Production Systems of the Leibniz Universität Hannover and the three greenhouse experiments (GH1-3) at the Department of Plant Propagation of the Leibniz Institute of Vegetable and Ornamental Crops. The increases in fresh and dry weights during temperature treatment were determined to obtain general information on biomass production at the differentiated temperatures. Since dry and fresh weight production responded in a similar manner to cv and temperature, only the dry weight production is shown in **Figures 1 - 5**. The *Petunia hybrida* cvs displayed highly specific growing rates, measured as dry weight increases after 4 or 5 weeks, independent of temperature treatment and experimental compartments.

In the climate chamber experiment (CC: **Figure 1 A; 2 A; 3 A, C**), Fam LB, UB and Fam LDV, the cvs with the highest dry weight increase after five weeks accumulated under both temperature treatments more than twice as much dry weight as SwSu Will, the cv with the lowest dry weight accumulation (**Figure 1 A**). In response to chilling treatment, the dry weights of all cvs were significantly reduced, with the exception of Com Fam EP and Fam F. A further experiment was conducted in greenhouse cabins (GH1: **Figure 1 B; 2 B; 3 B, D**). The cultivar-specific differences in dry weight accumulation were even bigger than in climate chamber (compare **Figure 1 A** and **B**). Fam LDV, Fam LB and UB produced the highest dry weight increases again. SwSu WE and SwSu Will showed the lowest dry weight production (**Figure 1 B**). The chilling response was in the most cvs stronger than in the climate chamber, and showed a significantly reduced dry weight production for all cvs (compare **Figure 1 A** and **B**).

Correlating the dry weight production at control and chilling temperature in CC, the regression line reflected a strong cultivar-specific correlation between growth at 11.5 °C and 15.75 °C (**Figure 2 A**). Nevertheless, Com Fam EP, Fam F and UB were situated above the regression line, whereas the other cvs were situated on or below the line. Correlating the dry weight productions at both temperatures in GH1 showed a slightly stronger cultivar-specific correlation between growth at chilling and control than in CC. In GH1, UB and Fam F were again placed above the regression line, indicating a slight above-average dry weight production under chilling compared to control (**Figure 2 B**). Additionally, Fam DB and SwSu Will were below the line as they were in CC.

In CC, also the growth ratios of Fam F, Com Fam EP, and UB showed only small chilling-induced growth restrictions (**Figure 3 A**), which was also reflected by lower absolute growth depressions (**Figure 3 C**). Higher growth depressions were found for the cvs with the lowest growth ratios as Fam DB, Com Fam HP and SwSu Bur. The absolute growth reduction of SwSu Will was smaller than in these three cultivars, while it still displayed a low growth ratio indicating a strong growth restriction. The two cvs positioned above the regression line in both experiments (**Figure 2 A, B**), Fam F, and UB, also displayed the highest growth ratios at chilling in GH1 (**Figure 3 B**), while SwSu Will and Fam DB, positioned below the regression line in both experiments, were positioned among the three cvs, with the lowest growth ratios. Whereas in CC, the cvs with high growth rates under exposure to chilling (**Figure 3 A**) simultaneously displayed the lowest growth depressions (**Figure 3 C**), in the GH1 there was no clear connection between growth ratio (**Figure 3 B**) and extent of growth depression (**Figure 3 D**). Furthermore, despite the significantly different growth ratios, the absolute growth depression of UB and SwSu Will were almost the same in both experiments.

Evaluation of the chilling stress on certain growth characteristics

To find indication, whether certain growth characteristics of the cvs at control or chilling temperature may determine the tolerance against chilling, we calculated the correlation between the different biomass production and development of structure parameter values determined at the specific temperature as independent variables and the ratio or absolute depression for the same values between 12 °C and 16 °C as dependent variables (**Table 2**). The data indicates that the relative robustness of growth against mild chilling is determined to a high extent by the absolute growth in terms of biomass production and branching rate (development of lateral shoots) in particular. Thus, ratios of fresh weight, shoot length and leaf number at 12 °C related to 16 °C are highly correlated to the fresh and dry weight production and to the shoot number produced at 16 °C. Especially the ratios of fresh weight were highly correlated with shoot number produced at 12 °C. Higher general growth in terms of fresh and dry weight production was also correlated to higher losses in dry weight in response to chilling, even though the reversal relationship was found for the absolute depression in leaf number. Nevertheless, against this background, the data provided an indication that particular cvs show specific responses to chilling which are not simply related to general growth characteristics.

Investigation of selected cultivars on mild chilling stress

A consecutive second greenhouse experiment (GH2) focused on a reduced number of cvs that reacted chilling-tolerant, chilling-sensitive or mediocre in the first two screenings (CC and GH1: **Figure 1** to **3** and **Table 1**). Focusing on these selected cvs reduced the overall variability in growth between the cvs so that the differences in tolerance were more pronounced. Thus, this experiment featured much less cultivar-specific growth differences at 16 °C, but displayed for all cvs, with exception of UB, a significant chilling response (**Figure 4 A**). Further, the distinct chilling reactions of UB and SwSu Will were confirmed. For both cvs, dry weight production did not vary significantly at control temperature, but differed under chilling. The growth ratio of SwSu Will was lower than the ratio of UB (**Figure 4 B**) and the growth depression stronger (**Figure 4 C**). Thus, due to the reduced number of cvs and the higher growth performance of UB under chilling, there was only a weak correlation of dry weight production at 12 °C and at 16 °C, while the data point of UB was above the regression line (**Figure 5**).

Comparison of growth parameter development of two different greenhouse experiments

Responses of particular structure parameters of the cvs to temperature were analyzed in two greenhouse experiments (GH1 and GH2). **Figure 6** features only the selected cvs, which were chosen for the analysis in GH2. In GH1, the shoot elongation growth varied between the cultivars and responded to chilling with significant decreases in all cvs (**Figure 6 A**). The production of new leaves on the main shoot showed cultivar-specific growth differences at both temperatures, and responded to chilling with significant decreases in the cvs except for Fam LB (**Figure 6 C**). The distinct growth

response of dry weight of UB and SwSu Will was not reflected by the elongation growth or the leaf development on the main shoot, both parameters did not vary significantly at chilling or control temperature between both cvs. However, the formation rate of new lateral shoots of the main shoot displayed the strongest cultivar-specific variations (**Figure 6 E**). The more dry-weight-producing cvs Fam LB and UB developed at both temperatures considerably more lateral shoots than the poor dry-weight-producing cv SwSu Will. Nevertheless, only Com Fam HP responded to chilling with a decrease in number of new shoots. In GH2, the shoot elongation was generally lower, which leads to smaller cv-differences at control temperature and no significant differences under chilling (**Figure 6 B**). However, all cvs still responded to chilling with a decrease of shoot length growth. The leaf development showed no cultivar-specific growth differences at both temperatures, whereas all cvs reacted to chilling with a reduced leaf production (**Figure 6 D**). GH2 proved that the distinct growth response of dry weight of UB and SwSu Will was not reflected by the elongation growth or the leaf development on the main shoot. Likewise, in GH1, both parameters did not vary significantly at chilling or control temperature in both cvs. Also in GH2, the formation rate of new lateral shoots displayed strong cultivar-specific differences, with higher rates for UB and Com Fam HP at both temperatures compared to GH1 (**Figure 6 F**). The chilling effect on branching was just marginal. Only in Com Fam HP, the number of newly developed shoots was significantly reduced. The findings of the first experiment that Fam LB and UB developed at both temperatures considerably more lateral shoots than SwSu Will could be reproduced.

Introduction of growth performance indices

For final comparison of the five cvs which were used in the three above-shown experiments CC, GH1 and GH2 and in an additional third greenhouse experiment, GH3, their indices for growth performance (GI), chilling performance (CPI) and chilling tolerance (CTI) were calculated based on dry weight production (**Table 3**). In addition to the mean values over the four experiments, SEs were calculated as indicators for the environmental stability of the growth and chilling reaction. GI and CPI reflect the relative growth performance of each cv in relation to the highest-performing cv at control temperature or chilling, respectively. The CTI describes the individual robustness of the specific cvs to the exposure to chilling. The highest GI and CPI and therefore the highest dry weight production at both temperatures compared to the pool of cvs, displayed Fam LB, but combined with the second lowest CTI. UB showed the second highest GI and CPI, but the highest CTI. However, regarding the individual growth robustness against chilling, measured by CTI, Fam LB, Com Fam HP and Com Fam EP differed only marginally. The biggest difference in the individual dry weight production response to chilling was found between UB and SwSu Will.

5 DISCUSSION

Producing thermophilic ornamentals like petunia at reduced temperatures could save a lot of heating energy, as long as the produced species would show a similar growth performance at sub-optimal temperatures compared to common cultivation temperatures. Growth of *Petunia hybrida* cvs is impacted to a varying degree by sub-optimal temperatures, depending on their genotype. For this study we investigated putatively chilling-tolerant, and -sensitive reacting cvs to evaluate if the degree of susceptibility to chilling can be connected with changes in development of specific growth parameters. Since especially the first weeks of cultivation before flowering during winter account for the highest heating demand, we investigated the vegetative growth only.

The applied temperatures of 12 °C compared to 16 °C (with two K day/night difference) seem appropriate as testing system for investigations of mild chilling tolerance in petunia. The data indicates that the variation in vegetative growth among commercial petunia cvs at sub-optimal temperatures close to 12 °C is to a high extent related to their general growth characteristics (**Figure 1**). However, selected cvs displayed specific chilling responses which reflect a variation in chilling tolerance being independent of the general growth capacity (**Table 3**).

The ten analyzed cvs showed high diversity in dry weight production (**Figure 1**) and dry weight production was reduced by chilling. However, the cultivar-specific differences between fast and slowly growing cvs were more pronounced than individual chilling-induced growth depressions. Interestingly, particular the cv but also the temperature effects on growth were more pronounced when greenhouse cultivation allowed some fluctuation of temperature and irradiance compared with stable climate chamber conditions (**Table 1, Figure 1, Figure 3**). The variation in growth under chilling was strongly determined by the general growth performance of the cvs as reflected by the high positive correlation between dry weight production at chilling and control temperature (**Figure 2**). Nevertheless, plotting of individual cvs (**Figure 2**) and the calculation of the ratio of dry weight production at 12 °C versus 16 °C indicated cultivar-specific differences in the chilling response (**Figure 3 A, B**). Focusing on fewer cvs and thus reducing the general variation in growth (**Figure 4, Figure 5**) further highlighted these differences. It also confirmed that the cv UB shows better growth under chilling compared to SwSu Will (**Figure 4 A**) and revealed that a higher growth robustness of UB against chilling (**Figure 4 B, Figure 5**). Whereas calculation of the 12/16 °C ratio gave relatively stable results for UB versus SwSu Will over four experiments (**Figure 3 A, B, Figure 4 B, Table 3**), the absolute growth depression in terms of dry weight (**Figure 3 C, D, Figure 4 C**) was highly variable between individual experiments. This may be based on the fact, that the absolute depression is the outcome of interaction between two factors, the chilling tolerance and the general growth performance of the cv (see the correlation between dry weight depression and fresh and dry weight production at 16 °C in **Table 3**).

The positive correlation of fresh weight ratio with the branching rates at both temperatures suggests that cvs that develop high numbers of lateral shoots may be less susceptible to chilling (**Table 2**). In contrast to the shoot elongation and the development of new leaves, the branching rate in most cvs was only marginally affected by chilling, but displayed high cultivar-specific temperature-independent differences (**Figure 6**). Since the numbers of lateral shoots were correlated at both temperatures with several relative growth parameters (**Table 2**), a higher number of shoots may contribute to a good relative growth performance under chilling. Especially when shoot elongation growth and leaf development are much more disturbed than the branching rate, biomass production of cvs with a higher branching rate should be less affected by chilling. A recent detailed study of the sensitive cv SwSu Will revealed that mild chilling stress disturbs plant functional integrity at levels of plant hormones and carbohydrate metabolism (Bauerfeind et al., 2015). The metabolic data indicated that under mild chilling sugars accumulate in source leaves whereas carbohydrate transport to and/or carbohydrate utilization in the growing shoot apices are inhibited. A higher branching rate and thus a higher number of new lateral shoots implies also a higher number of growing points which provide large utilization sinks for carbohydrates being produced in the source leaves (Lieth et al., 1991). Therefore, a higher branching rate may reflect a generally different plant hormone homeostasis (e.g. of auxin) and by that altered carbohydrate flux situation (Mason et al., 2014), which may further contribute to chilling tolerance. Further, the higher number of shoots might increase the carbohydrate demand of the utilization sink tissues and probably increase the carbohydrate translocation from the source leaves. Thus, a detailed analysis of carbohydrate metabolism and plant hormone homeostasis and signaling in cvs with contrasting branching rate and chilling tolerance is necessary to elucidate such relationships. Nevertheless, a more complex branching habitus of a cv might be a usable first selection criterion, when screening a huge sets of *Petunia hybrida* cvs for chilling-tolerant cvs.

The specific indices, reflecting the growth at control (GI) and chilling (CPI) conditions and the growth robustness to chilling (CTI), suggest that variation in growth of commercial petunia cvs under the condition of mild chilling is to a great part determined by general growth performance. However, the data shows that chilling tolerance is not in all cases simply determined by the general growth. Further, relative growth under stress versus control conditions revealed distinct differences in chilling tolerance among the investigated cvs independent of general growth characteristics. GI and CPI may serve as indices to describe the growth of a particular cv at control temperature or chilling, respectively, in relation to the growth performance of the best growing cv of a certain pool. However, we propose CTI as stand-alone index to evaluate the individual reaction of a cv to chilling. Calculation of the SE for the different values over individual experiments or tests provides information on the environmental stability of growth performance, chilling performance and chilling tolerance of the analyzed cvs.

The vegetative growth of commercial *Petunia hybrida* cvs at sub-optimal temperatures in terms of dry weight production, shoot elongation and leaf development, but not branching of lateral shoots was reduced compared with control temperature but strongly determined by the general cv-specific growth potential. Furthermore, growth analyses suggest a higher chilling tolerance in cvs that develop more lateral shoots. This work suggests distinct indices to describe chilling performance and chilling tolerance of cvs. Finally, UB and SwSu Will seem suitable as models for further physiological and molecular investigations.

ACKNOWLEDGEMENTS

We thank Sabine Czekalla (IGZ) and Herbert Geyer (LUH) for their exceptional technical support in cultivating the petunias and evaluating the growth parameters. Further, we thank Kathrin Schulz for substrate analyses and Dr. Siegfried Zerche (both IGZ) for helpful advices regarding the fertilization of plants. We thank Prof. Dr. Margrethe Serek (LUH) for her scientific support. We thank Beate ter Hell and Dr. Dirk Ludolph (Lehr- und Versuchsanstalt für Gartenbau Ahlem) for their support in developing the chilling test conditions and Selecta Klemm GmbH & Co. KG (Stuttgart) for recommending the cvs and providing rooted cuttings.

The work was funded by the BMBF (Federal Ministry of Education and Research of the Federal Republic of Germany) within the BMBF-AgroClustEr: WeGa - Horticulture Research Network - Product und Production safety in intensive Plant production systems and further supported by ministries of the state of Brandenburg, the Free State of Thuringia and the Federal Republic of Germany.

5 REFERENCES

Allen, D. J., and Ort, D. R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends Plant Sci.* 6, 36–42. doi:10.1016/S1360-1385(00)01808-2.

Bauerfeind, M. A., Winkelmann, T., Franken, P., and Druege, U. (2015). Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress. *Front. Plant Sci.* 6:583. doi:10.3389/fpls.2015.00583.

BMEL, Bundesministerium für Ernährung und Landwirtschaft (2014). Verbrauch von Blumen und Zierpflanzen. *Der Gartenbau Deutschl. - Daten und Fakten*, 30 – 31. Available at: http://berichte.bmelv-statistik.de/GBB-0000100-2012.pdf. Accessed 05 Mar 2016.

Elings, A., Kempkes, F. L. K., Kaarsemaker, R. C., Ruijs, M. N. A., van de Braak, N. J., and Dueck, T. A. (2005). The energy balance and energy-saving measures in greenhouse tomato cultivation. *Acta Hortic.* 691, 67–74. doi:10.17660/ActaHortic.2005.691.5.

Gabot.de (2012). GABOT: Gartenbau: Energiekrise bedroht Konkurrenzfähigkeit. Available at: http://www.gabot.de/index.php/News-Details/52/0/?&tx_ttnews[tt_news]=226333tx_ttnews[backPid]= 1&cHash=6cffc84d7d Accessed May 7, 2016.

Kaczperski, M. P., Carlson, M. G., and Karlsson, M. G. (1991). Growth and development of *Petunia* × *hybrids* as a function of temperature and irradiance. *J. AMER. Soc. Hort. Sci.* 116, 232–237.

Kelly, R. O., Deng, Z., and Harbaugh, B. K. (2007). Evaluation of 125 petunia cultivars as bedding plants and establishment of class standards. *Horttechnology* 17, 386–396.

Lieth, J. H., Merritt, R. H., and Kohl, H. C. (1991). Crop productivity of petunia in relation to photosynthetically active radiation and air temperature. *J. AMER. Soc. Hort. Sci.* 116, 623–626.

Lucau-Danila, A., Toitot, C., Goulas, E., Blervacq, A. S., Hot, D., Bahrman, N., Sellier, H., Lejeune-Hénaut, I., and Delbreil, B. (2012). Transcriptome analysis in pea allows to distinguish chilling and acclimation mechanisms. *Plant Physiol. Biochem.* 58, 236–244. doi:10.1016/j.plaphy.2012.07.012.

Mahajan, S., and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.* 444, 139–158. doi:10.1016/j.abb.2005.10.018.

Mason, M. G., Ross, J. J., Babst, B. a., Wienclaw, B. N., and Beveridge, C. A. (2014). Sugar demand, not auxin, is the initial regulator of apical dominance. *Proc. Natl. Acad. Sci.* 111, 6092–6097. doi:10.1073/pnas.1322045111.

Pennycooke, J. C., Cox, S., and Stushnoff, C. (2005). Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (*Petunia x hybrida*). *Environ. Exp. Bot.* 53, 225–232. doi:10.1016/j.envexpbot.2004.04.002.

Sanghera, G. S., Wani, S. H., Hussain, W., and Singh, N. B. (2011). Engineering cold stress tolerance in crop plants. *Curr. Genomics* 12, 30–43. doi:10.2174/138920211794520178.

Schuch, I., Dannehl, D., Miranda-Trujillo, L., Rocksch, T., and Schmidt, U. (2014). ZINEG project - energetic evaluation of a solar collector greenhouse with above-ground heat storage in Germany. *Acta Hortic.* 1037, 195–201. doi:10.17660/ActaHortic.2014.1037.20.

Warner, R. M. (2010). Temperature and photoperiod influence flowering and morphology of four *Petunia spp*. *HortScience* 45(3), 365–368.

6 FIGURES

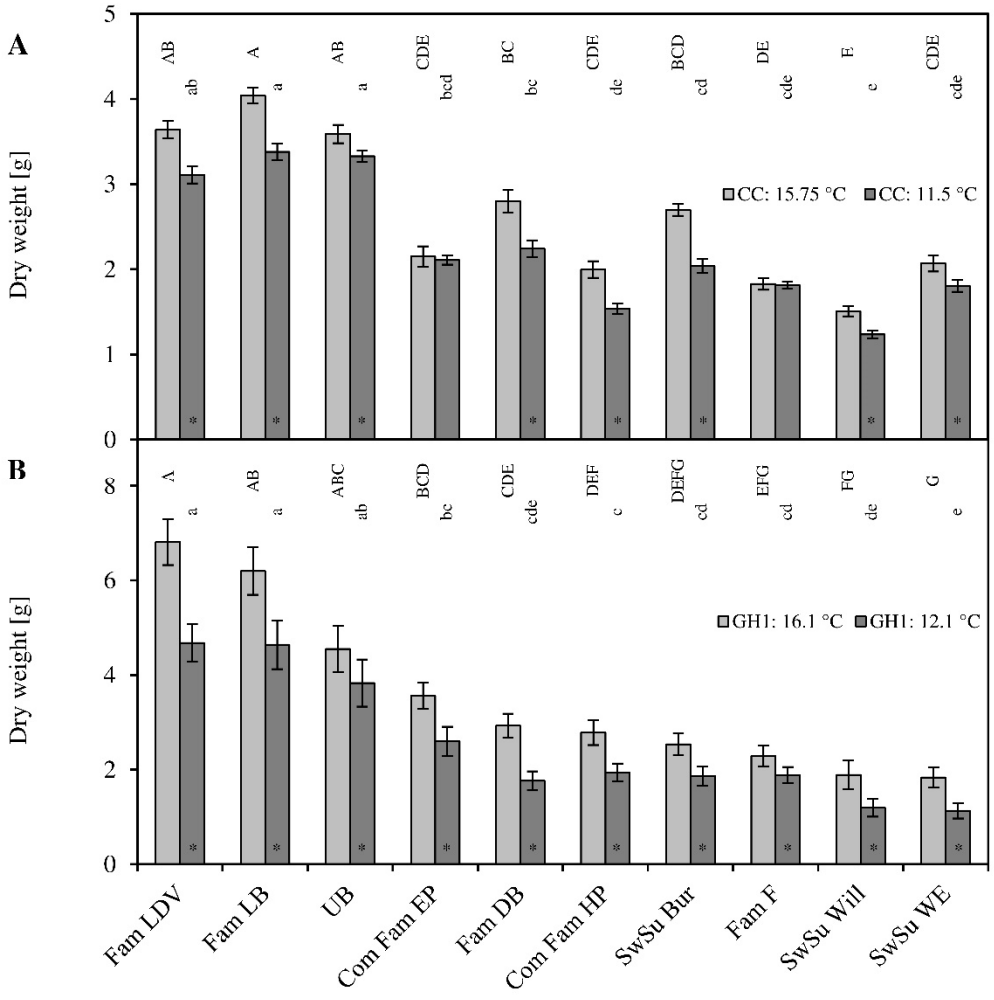


Figure 1 | Impact of chilling on dry weight production in climate chamber and greenhouse. Dry weight increases after four weeks of temperature treatment. (A) dry weight increases in climate chambers after five weeks (CC, control temperature mean: 15.75 °C, chilling mean: 11.5 °C); (B) dry weight increases in greenhouse cabins after four weeks (GH1, control temperature mean: 16.1 °C, chilling mean: 12.1 °C). (GH1: $n = 24$; CC: $n = 18$; data are means ± SE; different characters indicate significant differences between the cvs, $P < 0.05$; asterisks indicate significant temperature effects within one cv, * $P \leq 0.05$).

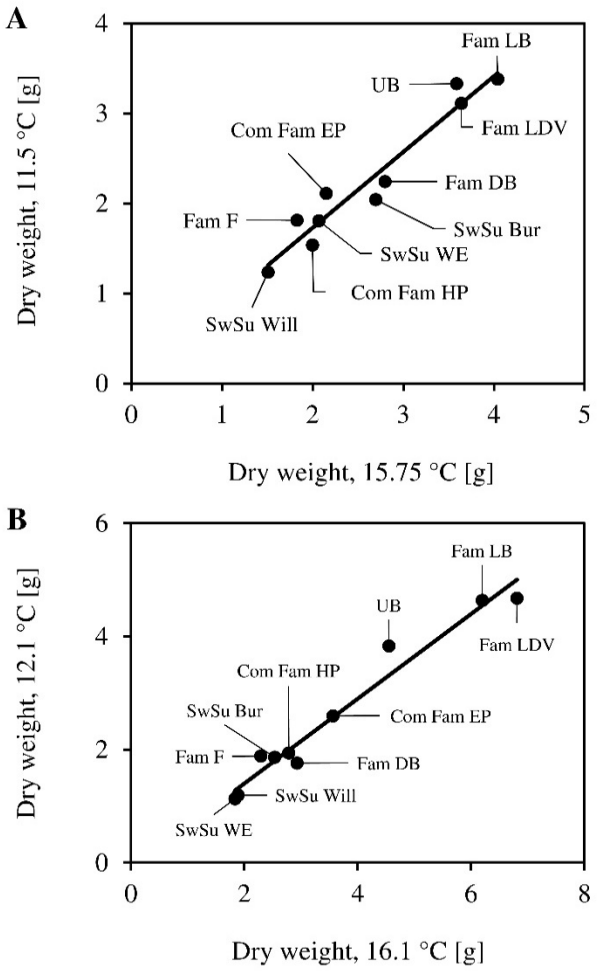


Figure 2 | Dry weight increases under chilling vs. increases at control temperature. Correlations of mean dry weight production per cv at chilling temperature with dry weight production at control temperature. (A) in climate chambers after five weeks (CC, control temperature mean: 15.75 °C, chilling mean: 11.5 °C; y = 0.7486x – 0.0968; R^2 = 0.9624); (B) in greenhouse cabins after four weeks (GH1; control temperature mean: 16.1 °C, chilling mean: 12.1 °C; y = 0.8425x – 0.0427; R^2 = 0.9357).

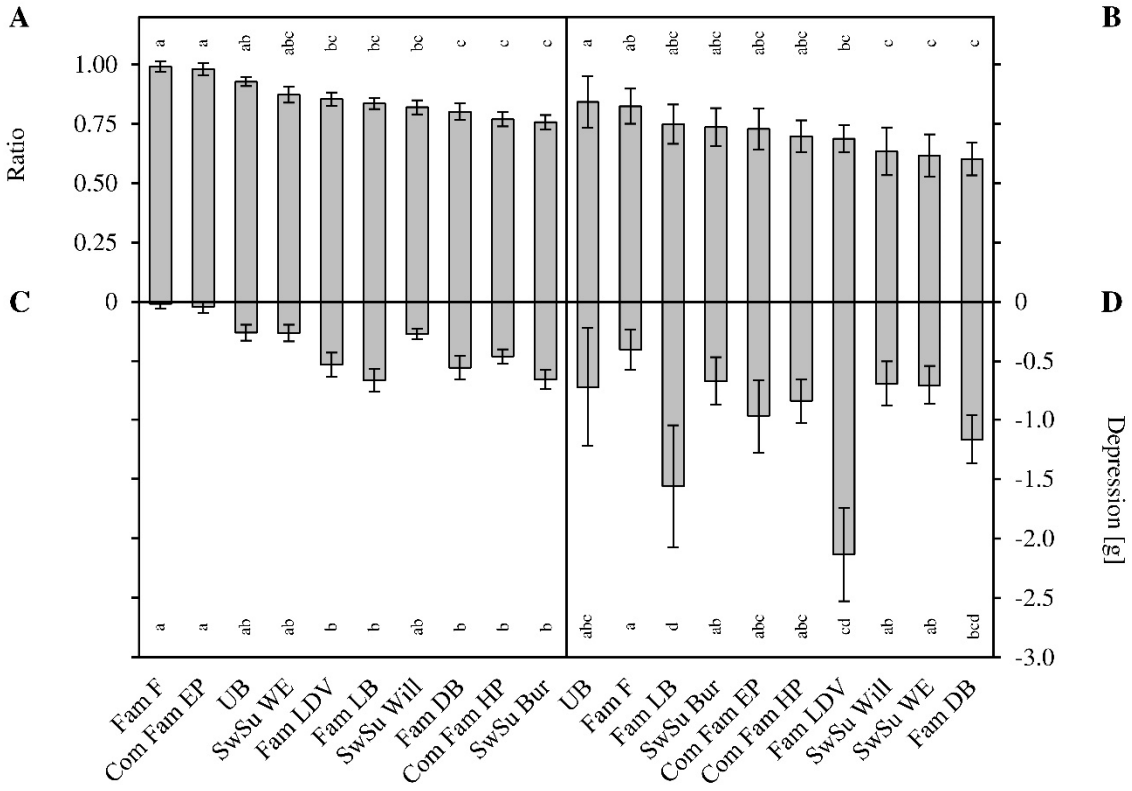


Figure 3 | Growth ratio and absolute growth depression. Growth ratios (dry weight production under chilling divided by production at control temperature) compared to the absolute growth depressions (difference of dry weight production under chilling and at control temperature) caused by exposure to chilling. (A) growth ratio in climate chambers after five weeks (CC, control temperature mean 15.75 °C, chilling mean 11.5 °C); (B) growth ratio in greenhouse cabins after four weeks (GH1, control temperature mean: 16.1 °C, chilling mean: 12.1 °C); (C) absolute growth depression in climate chambers; (D) absolute growth depression in greenhouse cabins. (GH1: n = 24; CC: n = 18; data are means ± SE; different characters indicate significant differences between the cvs, $P < 0.05$; asterisks indicate significant temperature effects within one cv, * $P \leq 0.05$).

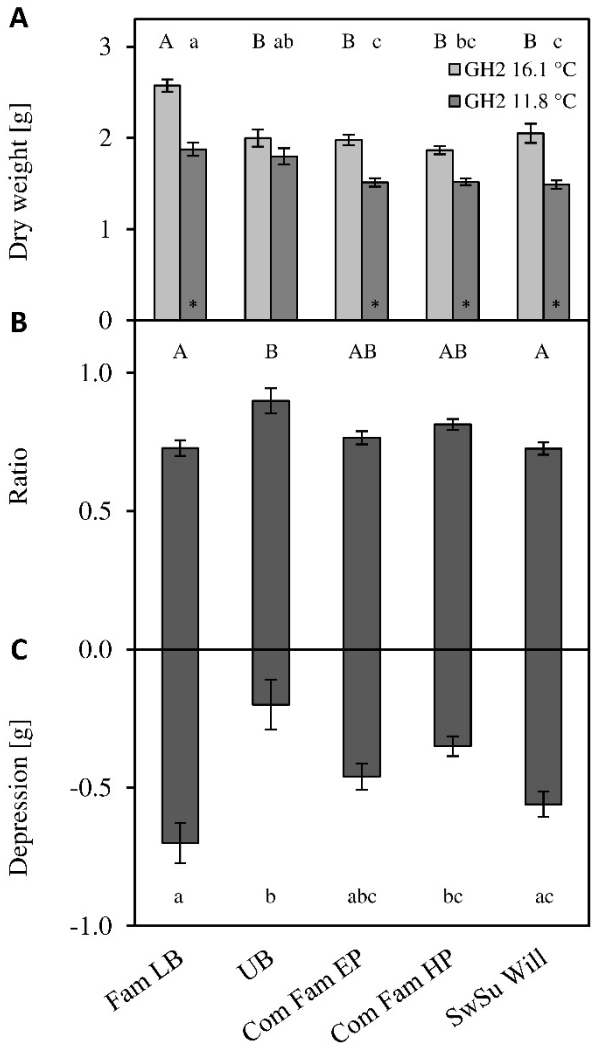


Figure 4 | Impact of chilling on dry weight production of selected cvs. Impact of chilling on absolute dry weight production, growth ratios (dry weight production under chilling divided by production at control temperature) and on dry weight depression (difference of dry weight production under chilling and at control temperature) of five selected cvs in a second greenhouse experiment (GH2). Dry weight increases after differentiated temperature treatment. (A) dry weight increases after four weeks; (B) growth ratio; (C) absolute growth depression. (GH2, control temperature mean: 16.1 °C, chilling mean: 11.8 °C; n = 16; data are means ± SE; different characters indicate significant differences between the cvs, $P < 0.05$; asterisks indicate significant temperature effects within one cv, * $P \leq 0.05$).

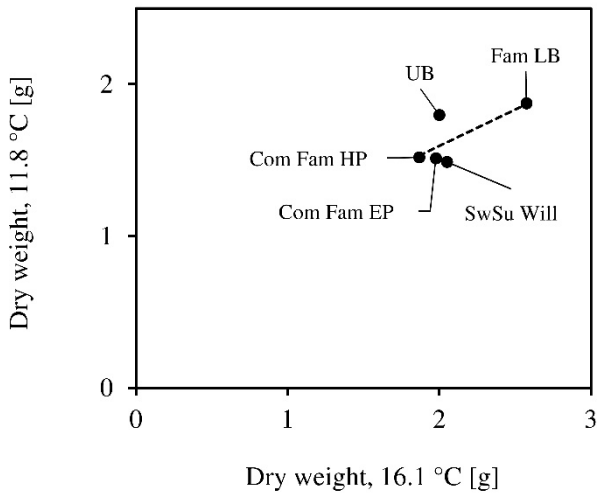


Figure 5 | Dry weight increases of selected cvs under chilling vs. increases at control temperature. Correlations of mean dry weight production per cv at chilling temperature with dry weight production at control temperature in greenhouse cabins after four weeks (GH2, control temperature mean: 16.1 °C, chilling mean: 11.8 °C; $y = 0.4787x - 0.6364$; $R^2 = 0.5262$).

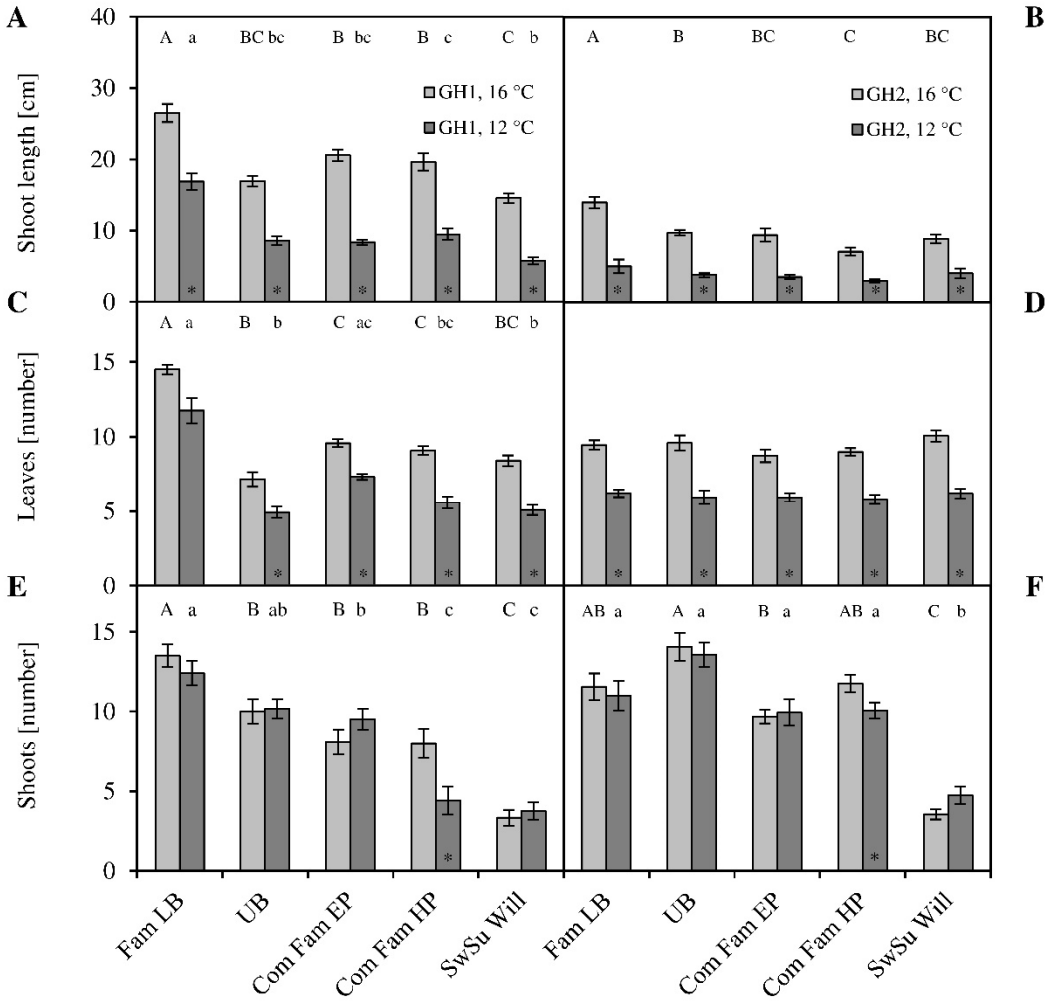


Figure 6 | Impact of chilling on growth parameters of selected cvs. Impact of chilling on shoot elongation, production of leaves and production of lateral shoots. Increases after four weeks of differentiated temperature treatment. (A) elongation growth of the main shoot (GH1, control temperature mean: 16.1 °C, chilling mean: 12.1 °C); (B) elongation growth of the main shoot (GH2, control temperature mean: 16.1 °C, chilling mean: 11.8 °C); (C) newly developed leaves (GH1); (D) newly developed leaves (GH2); (E) newly developed shoots (GH1); (F) newly developed shoots (GH2). (GH1: n = 24; GH2: n = 16; data are means ± SE; different characters indicate significant differences between the cvs, $P < 0.05$; asterisks indicate significant temperature effects within one cv, * $P \leq 0.05$).

Table 1 | Growth conditions of individual experiments. Conditions of the four experiments. (CC = climate chamber, LUH; GH1 - 3 = greenhouse cabins, IGZ; experiments GH2 and GH3 were conducted simultaneously as independent repetitions with plants derived from distinct shipments).

	CC		GH1		GH2		GH3	
	Control	Chilling	Control	Chilling	Control	Chilling	Control	Chilling
Duration of temperature treatment	5 weeks		4 weeks		4 weeks		4 weeks	
Average temperatures:								
Day	16.5 °C	12.5 °C	16.8 °C	13.1 °C	17.1 °C	13.0 °C	17.1 °C	13.0 °C
Daily min/max	-	-	15.1 °C/17.6 °C	11.8 °C/14.1 °C	14.8 °C/18.6 °C	10.8 °C/14.1 °C	14.8 °C/18.6 °C	10.8 °C/14.1 °C
Night	15.0 °C	10.5 °C	15.2 °C	11.0 °C	15.2 °C	10.6 °C	15.2 °C	10.6 °C
Daily min/max	-	-	14.6 °C/16.7 °C	10.0 °C/12.4 °C	14.6 °C/16.5 °C	10.2 °C/12.5 °C	14.6 °C/16.5 °C	10.2 °C/12.5 °C
24 h	15.75 °C	11.5 °C	16.1 °C	12.1 °C	16.1 °C	11.8 °C	16.1 °C	11.8 °C
Length of photoperiod	12 h		12 h		12 h		12 h	
Photosynthetic photon flux density	150 µmol/s*m²		day length extension by assimilation lighting		day length extension by assimilation lighting		day length extension by assimilation lighting	
Photosynthetic photon flux density of outside light (time frame)	(May 2011)		6.14 mol/d*m^2 (24 h mean, 29.03.2011 - 28.04.2011)		6.61 mol/d*m² (24 h mean, 01.02.2012 - 02.03.2012)			
Humidity	65%		79%	83%	61%	62%	61%	62%

Table 2 | Spearmans correlation coefficients: absolute production vs. ratio and growth depression under chilling. Correlations between the increases in fresh weight, dry weight, shoot number, shoot length and leaf number and the ratios (increase under chilling divided by increase at control temperature) and absolute growth depressions (difference of increase under chilling and at control temperature) of these parameters. (GH1; n = 10; data are means ± SE; bold numbers indicate significant correlations, $P \leq 0.05$).

	Ratio (12 °C/16 °C)					Growth depression (16 °C - 12 °C)				
Increases	**Fresh weight**	**Dry weight**	**Shoot number**	**Shoot length**	**Leaf number**	**Fresh weight**	**Dry weight**	**Shoot number**	**Shoot length**	**Leaf number**
Fresh weight 12 °C	0.62	0.42	0.47	**0.66**	0.42	-0.38	0.32	-0.36	-0.10	-0.24
Fresh weight 16 °C	**0.76**	0.42	0.16	**0.68**	**0.84**	-0.15	**0.72**	-0.10	0.18	**-0.64**
Dry weight 12 °C	0.56	0.30	0.42	0.61	0.44	-0.19	0.49	-0.31	0.07	-0.21
Dry weight 16 °C	**0.76**	0.42	0.16	**0.68**	**0.84**	-0.15	**0.72**	-0.10	0.18	**-0.64**
Shoots 12 °C	**0.87**	0.62	0.27	**0.72**	**0.78**	-0.49	0.36	-0.20	-0.19	-0.53
Shoots 16 °C	**0.78**	**0.79**	-0.27	**0.68**	**0.65**	**-0.65**	0.05	0.32	-0.31	-0.39
Shoot length 12 °C	-0.04	-0.24	-0.15	0.31	0.49	0.47	**0.74**	0.19	0.61	-0.08
Shoot length 16 °C	-0.08	-0.49	0.24	-0.21	0.39	0.14	0.60	-0.22	**0.70**	-0.36
Leaf number 12 °C	0.54	0.63	-0.36	0.49	0.34	-0.26	0.00	0.44	-0.07	0.28
Leaf number 16 °C	0.06	0.06	-0.03	0.19	0.26	-0.14	-0.10	0.09	-0.18	0.32

Table 3 | Growth indices. Growth index (GI), cold performance index (CPI) and cold tolerance index (CTI). Means of four experiments (CC, GH1, GH2 and GH3).

	GI		CPI		CTI	
Cultivar	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Fam LB	1.00	0.00	0.99	0.04	0.76	0.04
UB	0.78	0.07	0.88	0.08	0.87	0.08
Com Fam EP	0.72	0.11	0.75	0.11	0.81	0.11
Com Fam HP	0.57	0.09	0.58	0.11	0.78	0.10
SwSu Will	0.48	0.12	0.44	0.12	0.70	0.12

2.2 Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 July 2015
doi: 10.3389/fpls.2015.00583

Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress

*Martin Andreas Bauerfeind[1], Traud Winkelmann[2], Philipp Franken[1] and Uwe Druege[1]**

[1] Department of Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops, Erfurt, Germany, [2] Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hannover, Germany

OPEN ACCESS

Edited by:
Elena Prats, Consejo Superior de Investigaciones Científicas, Spain

Reviewed by:
Esther M. Gonzalez, Universidad Publica de Navarra, Spain
Andreas Savvides, AgriSearch Innovations Ltd., Cyprus

***Correspondence:**
Uwe Druege, Department of Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops, Kuehnhaeuser Straße 101, 99090 Erfurt, Germany
druege@erfurt.igzev.de

Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: *30 April 2015*
Accepted: *13 July 2015*
Published: *28 July 2015*

Citation:
Bauerfeind MA, Winkelmann T, Franken P and Druege U (2015) Transcriptome, carbohydrate, and phytohormone analysis of Petunia hybrida reveals a complex disturbance of plant functional integrity under mild chilling stress. Front. Plant Sci. 6:583. doi: 10.3389/fpls.2015.00583

Cultivation of chilling-tolerant ornamental crops at lower temperature could reduce the energy demands of heated greenhouses. To provide a better understanding of how sub-optimal temperatures (12°C vs. 16°C) affect growth of the sensitive *Petunia hybrida* cultivar 'SweetSunshine Williams', the transcriptome, carbohydrate metabolism, and phytohormone homeostasis were monitored in aerial plant parts over 4 weeks by use of a microarray, enzymatic assays and GC-MS/MS. The data revealed three consecutive phases of chilling response. The first days were marked by a strong accumulation of sugars, particularly in source leaves, preferential up-regulation of genes in the same tissue and down-regulation of several genes in the shoot apex, especially those involved in the abiotic stress response. The midterm phase featured a partial normalization of carbohydrate levels and gene expression. After 3 weeks of chilling exposure, a new stabilized balance was established. Reduced hexose levels in the shoot apex, reduced ratios of sugar levels between the apex and source leaves and a higher apical sucrose/hexose ratio, associated with decreased activity and expression of cell wall invertase, indicate that prolonged chilling induced sugar accumulation in source leaves at the expense of reduced sugar transport to and reduced sucrose utilization in the shoot. This was associated with reduced levels of indole-3-acetic acid and abscisic acid in the apex and high numbers of differentially, particularly up-regulated genes, especially in the source leaves, including those regulating histones, ethylene action, transcription factors, and a jasmonate-ZIM-domain protein. Transcripts of one Jumonji C domain containing protein and one expansin accumulated in source leaves throughout the chilling period. The results reveal a dynamic and complex disturbance of plant function in response to mild chilling, opening new perspectives for the comparative analysis of differently tolerant cultivars.

Keywords: ***Petunia*, cold, carbohydrate metabolism, sugars, invertase, gene expression, microarray, plant hormones**

Introduction

Petunia (*Petunia hybrida*) is one of the economically most important ornamental crops in Europe and North America. In Germany, it is one of the top ten bedding plant species. In 2012, German ornamental crop-producing companies produced more than 43 million petunias (Bundesministerium für Ernährung, Verbraucherschutz und Landwirtschaft, 2013). In moderate and northern climates, bedding crops like petunia are mainly produced during the winter months and early spring. Thus, heating of greenhouses is crucial to maintain optimal conditions for production of these thermophile plants. Exemplary, for greenhouse cultivation of tomato, Elings et al. (2005) reported energy savings of 16%, obtained from a 2 K reduction of mean cultivating temperature. Consequently, production at lower temperatures could save a lot of energy and significantly reduce emission of greenhouse gases, contributing to a more sustainable production. However, production at sub-optimal temperature would lead to retarded plant growth and development, which would extend production time and thus increase energy consumption again. For that reason, cultivars are needed which show a minimum growth depression when cultivated at reduced temperature compared to optimal conditions. Better understanding of how mild chilling stress in response to slightly reduced temperatures affects plant growth would ease the breeding of such cultivars. In the context of the presented research, mild chilling is understood as a slight reduction of temperature below commonly used production temperatures, which provokes a significant decrease in growth without causing visible cold damage.

Previous research covered cold and freezing reactions of *Arabidopsis thaliana* (Zhu et al., 2007; Guy et al., 2008; Shi et al., 2012) and also of other plant species (Sasaki et al., 2001; Zhang et al., 2012). However, only few studies have addressed plant responses to mild chilling at slightly reduced temperatures (Usadel et al., 2008; Yamori et al., 2009). Although *Arabidopsis* is the most advanced and best investigated model plant species, it does not perfectly represent all other species (Schwarz-Sommer et al., 2003). The genus *Petunia* serves as outstanding model system regarding ecological niches and diversity of forms and thus covers a more diverse range than *Arabidopsis* (Gerats and Vandenbussche, 2005). While phenotypical studies regarding growth at sub-optimal temperatures exist (Warner, 2010), chilling responses of petunia at the level of molecular physiology have not been analyzed yet.

The chilling response of the plant phenotype reflects changes in the habitus like a reduced growth performance, which are the consequences of a disturbed plant metabolism and of the acclimation to the changed environment at transcriptional or post-transcriptional level. Usadel et al. (2008) showed for *Arabidopsis* rosettes that already small decreases of the ambient temperature cause changes in metabolism and expression of genes. Interestingly, this response to mild chilling revealed a remarkable similarity to the response to cold temperature of 4°C, but with a smaller extent of the reaction. The synthesis of cryoprotectants and stress metabolites was enhanced and leaf protein synthesis was up-regulated, accompanied by an increase of the protein content (Usadel et al., 2008). In the youngest mature source leaves of tomato, increased sugar, and starch levels were observed in response to a moderate reduction of temperature (day/night temperature: 16/14°C vs. 25/20°C; Venema et al., 1999). While especially at very low temperatures increases of osmolytes may protect cells from freezing (Mahajan and Tuteja, 2005), reduced export of carbohydrates might be responsible for reduced growth at chilling temperatures. Furthermore, particularly in thermophile plants, photosynthesis is considerably reduced after chilling due to impaired redox and circadian regulation (reviewed in Allen and Ort, 2001). This effect might underlie the impact of chilling on carbohydrate metabolism. Studies revealed that cold-induced inhibition of sucrose synthesis causes a phosphate-limitation of photosynthesis (Hurry et al., 2000). Cold acclimation of *Arabidopsis* leaves seems to be triggered by low phosphate, which induces changes in Calvin cycle enzymes like increased expression of Rubisco and sucrose biosynthesis enzymes (Hurry et al., 2000). Moreover, Usadel et al. (2008) reported a coordinated repression of some genes, responsible for starch and sucrose breakdown. Thus, transcript levels for vacuolar invertase as well as total invertase activity were reduced at low temperature.

Some phytohormones such as auxins, abscisic acid (ABA) and jasmonic acid (JA) are known to be involved in plant reactions to abiotic stress resulting from low temperatures. Auxin is a key regulatory phytohormone in plant growth and development, while indole-3-acetic acid (IAA) constitutes the most important physiologically active fraction. It plays an important role in plant reactions to environmental changes. Changed plant growth and development in response to cold may be linked to an altered intracellular homeostasis of auxins, which is regulated by local auxin gradients (reviewed in Rahman, 2013). Studies in *Arabidopsis* roots suggest that cold exposure affects auxin transport rather than auxin signaling. It is assumed that the intracellular trafficking of auxin efflux carriers is inhibited under cold stress (Shibasaki et al., 2009). Another important stress related phytohormone that plays a crucial role for chilling reaction is ABA. ABA synthesis seems to be essential for acclimation-induced chilling tolerance in maize seedlings (Anderson et al., 1994). Ntatsi et al. (2013) reported that tolerance of tomato to mild chilling stress was dependent on ABA biosynthesis, even though ABA accumulation itself seemed not to be essential for the acclimation mechanism. The biosynthesis and signaling of JA seems also to be important for maintaining normal physiological functions under cold stress (Du et al., 2013a; Hu et al., 2013).

Among the diverse mechanisms, providing plant plasticity toward a changing environment, the regulation of gene expression plays an important role in the genetic potential of a plant to cope with environmental stresses. In this context, the MYC-like transcription factor (TF) ICE1 from the bHLH family has a crucial control function in cold tolerance in *Arabidopsis*. ICE1 regulates the expression of several TFs that repress or activate further downstream cold-responsive genes (Chinnusamy et al., 2003; Lee et al., 2005). Other research disclosed an important role of the CBF family of TFs for adaptive changes of expression and metabolism in response to small decreases

of temperature (Usadel et al., 2008). Recent studies proofed the important role of constitutive CBF expression for freezing tolerance also in petunia (Walworth et al., 2014). However, transcriptional profiling of petunia seedlings in response to cold at 2°C indicates that besides the CBF pathway diverse other regulatory pathways may exist that regulate the cold stress response (Li et al., 2015). Considering that the molecular and physiological response of petunia to water deficit follows both a severity- and time-dependent pattern (Kim et al., 2012), the transcriptional response to mild chilling may differ from the cold response to temperatures close or even below 0°C.

The molecular and physiological response of *P. hybrida* to mild chilling at the levels of carbohydrate metabolism, phytohormone homeostasis, and the transcriptome is unknown. In the present study, we monitored such responses in the chilling-sensitive cultivar 'SweetSunshine Williams.' This cultivar shows a strong growth depression during a period of 4 weeks under a temperature of 12°C when compared to 16°C, a temperature frequently used for greenhouse production of petunia in Germany. We followed the hypothesis that the growth depression is related to phase-specific changes in plant carbohydrate metabolism and/or phytohormone homeostasis. Therefore, we monitored carbohydrate levels and invertase activity by enzymatic assays and the phytohormones IAA, ABA, and JA by gas chromatography-tandem mass spectrometry (GC-MS/MS) in different aerial plant parts after differentiation of temperature. Furthermore, to obtain a comprehensive picture of the acclimation at gene expression level, we analyzed the transcriptome by use of a specific petunia microarray. This microarray provides 24,816 unique, non-redundant annotated sequences (Breuillin et al., 2010; Ahkami et al., 2014).

Materials and Methods

Plant Material and Sub-Optimal Temperature Treatment

All experiments were carried out with the chilling-sensitive *P. hybrida* cultivar 'SweetSunshine Williams' ('Williams'), which shows a significant reduction in dry weight production, when exposed to sub-optimal temperature. As sub-optimal temperature, an average day temperature of 12°C was chosen and compared to an average day temperature of 16°C, which is commonly used in German greenhouse production, as control. With the cultivar 'Williams,' this decrease in temperature of 4 K causes a retarded growth without any visible damage due to cold stress. All plants, used for the experiments, were vegetatively propagated. Cuttings were rooted under greenhouse conditions. ED 73 petunia substrate was used for potting, but without slow-release fertilizers (Einheitserde Classic Tonsubstrat ED 73, +Fe, coarse, nutritive salt 1.0, without slow-release fertilizer (GEPAC LCD); Patzer GmbH & Co. KG, Sinntal, Germany). Salt and nutrient concentrations and pH of substrate were controlled during the experiments. Plants were fertilized with 0.15% Hakaphos spezial (16% N, 8% P_2O_5, 22% K_2O, 3% MgO, and micronutrients; COMPO GmbH Münster, Germany) once a week.

Detailed growth performance analysis was conducted under greenhouse conditions (control temperature, day/night average: 16.8°C/15.2°C; sub-optimal temperature, day/night average: 13.1°C/11.1°C; 12 h photoperiod with ±130 μmol m^{-2} s^{-1} photo-synthetic photon flux density). All other experiments were conducted under climate chamber conditions. Therefore, immediately after potting, rooted cuttings were transferred to a climate chamber to acclimate to control temperatures (day/night: 17°C/15°C) and to develop a good root system. Day length was fixed to a 12 h photoperiod, (photo-synthetic photon flux density: during first week 100 μmol m^{-2} s^{-1}, from second week onward 150 μmol m^{-2} s^{-1} (lighting: fluorescent tubes, FQ80W/865 HO Constant, Lumilux Cool Daylight, Osram, Germany), while the relative humidity was 65%. After 2 weeks of acclimation, half of the plants were moved to a climate chamber with identical conditions but under chilling exposure (day/night: 13°C/11°C). The 4-weeks period of cultivation focused on the vegetative growth of plants. To avoid interference with competing sinks, developing flower buds were continuously removed from the plants as soon as being visible during the course of the experiment.

Growth Performance Analysis and Collection of Samples

For determination of growth, the main shoots of 12 plants per treatment were marked at the time point of 0 DoT (days after differentiation of temperature). As growth parameters, the number of newly developed shoots, the increase in length of the main shoot, and the number of newly developed leaves on the main shoot were evaluated weekly over a period of 28 days.

Samples were collected from three plant organs for analyses. As sink tissue, the apex of the main shoot was harvested, including adjacent small leaves (total length <2 cm). For sampling of source tissue, leaf disks (50–120 mg fresh weight for carbohydrates and 200–250 mg for phytohormones) were excised with a cork borer from the middle of each leaf half of young, but fully expanded leaves. For phytohormone and gene expression analyses, the uppermost internode (for microarray only at 21 DoT) was additionally collected. The samples were immediately transferred to liquid nitrogen and stored at −80°C until purification and analysis. The samples for carbohydrates, enzymes, and microarray were collected at 6 h, for phytohormone analyses at 8 h after begin of the photoperiod. Samples for carbohydrate, enzyme and phytohormone analyses were collected at 0, 1, 2, 3, 7, 14, 21, and 28 DoT, samples for the microarray analysis at 1, 3, 7, 21 DoT. Since metabolic and hormone data of 2 DoT gave no information in addition to those of 1 and 3 DoT, these were not included in the results.

Analysis of Carbohydrates, Invertase Activity, and Phytohormones

Extraction, purification, and analysis of ABA, IAA, and JA by GC-MS/MS were performed as described by Ahkami et al. (2013). That protocol is a modification of the original protocol, described by Müller et al. (2002). To measure all three phytohormones, the 1 ml methanol, added to frozen samples for extraction, contained 10.3 pmol $(^2H)_2$-IAA, 10.3 pmol $(^2H)_6$-ABA and 27.8 pmol

(^{2}H)$_6$-JA as internal standards. Gas chromatography and mass spectrometry settings for IAA, ABA, and JA were applied as described by Ahkami et al. (2013), Ntatsi et al. (2013), and Rasmussen et al. (2015). Ten biological replicates were analyzed per each treatment and date. Fructose, glucose, sucrose were extracted with 80°C aqueous ethanol. The sugars in the extract and starch in the extraction residue were analyzed in 96-well standard microplates using enzymatic assays as described by Hajirezaei et al. (2000) and Klopotek et al. (2010), using 12 biological replicates per treatment and date. Preparation and analysis of samples for invertase activities were conducted as described by Hajirezaei et al. (1993, 2000), using 10 biological replicates per treatment and date.

Statistical Analyses

To execute statistical analyses, the STATISTICA software package was used[1] [StatSoft, Inc. (2011); STATISTICA (data analysis software system), version 10]. The metabolite data were statistically analyzed by applying ANOVA in combination with student's *t*-test, if normal distribution within the groups and variance homogeneity were met. Normal distribution was tested by Kolmogorov–Smirnov test and variance homogeneity by Levene's test. If these assumptions were not fulfilled, a non-parametric test for comparing means (Mann–Whitney-*U* test or Kruskal–Wallis test) was applied and tested for significance (Ahkami et al., 2013). Significant differences at a level of $P \leq 0.05$ were marked by asterisks in the respective figures.

Microarray Hybridization and Statistical Analysis

In order to identify genes with altered expressions in response to sub-optimal temperatures, a petunia-specific microarray was hybridized, which carries 24,816 unigene annotated sequences (Ahkami et al., 2014) and was first described by Breuillin et al. (2010). RNA was extracted from three replicates by the QIAGEN kit (Qiagen, Hilden, Germany). Each replicate was a pooled sample of four individual plants. For extraction, RNA was treated with DNase, following the Qiagen protocol. A minimum of 500 ng total RNA per sample was required for array hybridization, performed by Oaklabs (OakLabs GmbH, Hennigsdorf, Germany). Normalization of the whole set of data was conducted by Oak Labs using the Quantil-normalization according to Bolstad et al. (2003). For analysis of each time point (1, 3, 7, and 21 DoT), expression values derived from samples grown at sub-optimal temperature (12°C) and at control temperature (16°C) were compared. *M*-values (Log_2 of ratios) were used to demonstrate the intensity of up- or down-regulation of genes under the exposure to chilling. $Log_2 > 1$ was defined as up-regulated, $Log_2 < -1$ was defined as down-regulated. For identification of statistically significant up- or down-regulated genes, a Rank Product online-analysis[2] was carried out (Breitling et al., 2004; Laing and Smith, 2010). Hereby, a pfp (probability of false prediction) value threshold <0.15 was applied to identify statistically significant differentially expressed genes. Expression graphs were created with Genesis, software version 1.7.6. (Sturn et al., 2002). Information regarding TFs in *Arabidopsis thaliana* was provided by the Stress Responsive Transcriptions Factor Database (STIFDB[3]; Naika et al., 2013).

Results

Growth Response to Mild Chilling

The chilling-sensitive *P. hybrida* cultivar 'Williams' reacted to the reduction of mean ambient temperature by 4 K with a strong decrease in growth and a retarded development. After 28 days, shoot dry and fresh weights were reduced by 37 and 43%, respectively (Supplementary Figure S1). This was associated with reductions of the elongation of the main shoot by 60% (Supplementary Figure S2A) and the number of newly developed leaves by 40% (Supplementary Figure S2C). However, the branching (Supplementary Figure S2B), i.e., the development of new shoots, was not affected by the chilling temperature.

Response of Carbohydrate Levels and Invertase Activities to Mild Chilling

Considering the crucial role of carbohydrates as protective molecules for plant survival at very low and freezing temperatures, we analyzed the concentrations of the most important sugars fructose (Fru), glucose (Glc), and sucrose (Suc) as well as starch in source leaves and in the shoot apex as important utilization sink. Samples were collected at midday, 6 h after the start of the photoperiod, at the expected climax of sugar concentrations (Nägele et al., 2010). A strong response of the carbohydrate levels to the chilling treatment was observed. However, the dynamic was different in source and sink tissues. During the first days of chilling exposure, concentrations of hexoses (Fru + Glc; **Figure 1A**), Suc (**Figure 1C**) and starch (**Figure 1E**) in source leaves increased significantly compared to the respective values of control plants. While hexose and Suc concentrations remained on significantly higher levels throughout the experiment, starch concentrations dropped down to values only slightly higher than in the control source leaves at 14–28 DoT with no significant difference at 21 DoT. In the shoot apex, especially hexoses, and starch displayed a chilling response that was composed of three phases, a fast, a midterm and a long-term response. At first, concentrations of hexoses (**Figure 1B**), Suc (**Figure 1D**), and starch (**Figure 1F**) increased under chilling stress similar to the response in the source tissue. However, the increase in the apex was smaller for all three fractions when compared to the leaves. After one week at sub-optimal temperature (midterm reaction), the concentrations of Suc and starch in the apex of chilled plants reached a steady state, which did not change thereafter, while hexose levels showed a slight increase. At control temperature, however, concentrations of hexoses and starch displayed a sharp increase during week three and four, resulting in significant higher concentrations at 28 DoT. This led to significant lower hexose and starch concentrations in the apex after prolonged

[1] www.statsoft.com

[2] http://strep-microarray.sbs.surrey.ac.uk/RankProducts/

[3] http://caps.ncbs.res.in/stifdb2/index.html

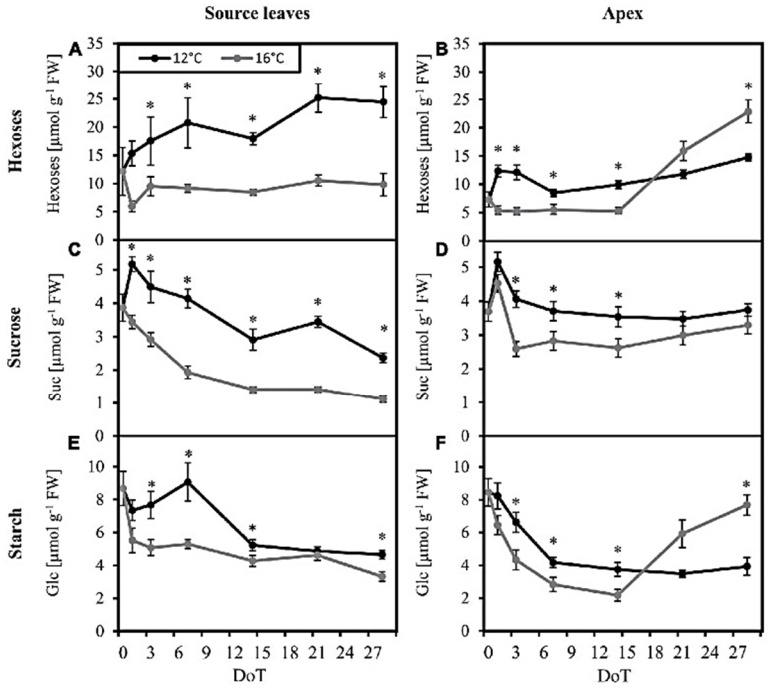


FIGURE 1 | Carbohydrate levels. Impact of sub-optimal temperature (black: 12°C, gray: 16°C) on carbohydrate levels. **(A)** source leaves, hexoses; **(B)** apex, hexoses; **(C)** source leaves, sucrose; **(D)** apex, sucrose; **(E)** source leaves, starch [specified in units of glucose (Glc)]; **(F)** apex, starch (specified in units of Glc). (DoT = days after differentiation of temperature; $n = 12$; data are means ± SE; asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

exposure to the chilling temperatures when compared to the control plants. Such relations were not observed in the source tissue.

While carbohydrate concentrations in the tissues at certain time points are snap-shots of current carbohydrate availability, carbohydrate ratios between source and sink and among each other may indicate bottlenecks of partitioning between carbohydrate fractions and between tissues at the two different temperatures. It becomes apparent from **Figure 2**, that cultivation at the sub-optimal temperature strongly reduced the ratio of carbohydrate levels between sink and source tissue when compared to the control plants. This difference started with sucrose (**Figure 2A**) at 7 DoT and was followed by the hexoses (**Figure 2B**) at 14 DoT. Exposure to sub-optimal temperature caused a transient reduction of the sucrose/hexose ratio in the apex until 14 DoT, which, however, from 21 DoT onward was followed by a reversed situation with higher sucrose/hexose ratios in the chilled plants (**Figure 2C**) To sum up, the carbohydrate levels and ratios indicate that the chilling treated plants accumulated sugars in the source leaves at the expense of carbohydrate transport to the shoot apex as utilization sink. In addition, from 21 DoT onward, Suc utilization in the shoot apex seemed to be inhibited as indicated by the increased Suc/hexose ratio in the apex.

Considering the changed carbohydrate levels and particularly the changed Suc/hexose equilibrium, we investigated the activities of cell wall bound (cwInv), cytoplasmic (cytInv), and vacuolar (vacInv) forms of invertase. In both, source and sink tissues, activities of cytInv (**Figures 3A,B**) and vacInv (**Figures 3C,D**) showed a slight, but mostly not significant increase of activities under chilling stress. In contrast, the activity of cwInv was significantly reduced under chilling stress, with a stronger absolute decrease of activity in the apex (**Figures 3E,F**).

Response of Phytohormone Levels to Mild Chilling

In order to unravel if the homeostasis of ABA, IAA, and JA in petunia is altered by exposure to sub-optimal temperature, concentrations of these phytohormones were monitored. Since the stem provides an important transport unit for phytohormones and, furthermore, IAA may control stem-elongation, the uppermost internode was analyzed in addition to the source leaves and the shoot apex. The mild chilling treatment had no measureable impact on levels of JA. For all three tissues, JA concentrations were very low. Actually, most values were below the limits of quantification of 1.5 pmol per injection (Rasmussen et al., 2015) for both treatments (data not shown). Therefore, an evaluation of JA homeostasis was

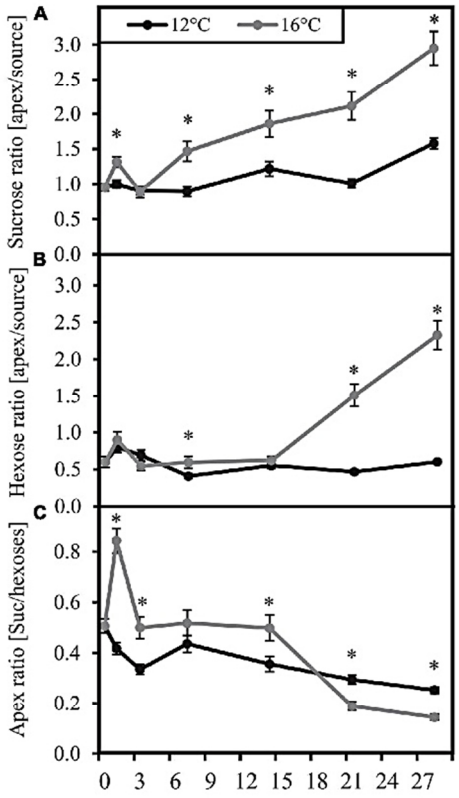


FIGURE 2 | Carbohydrate ratios. Ratios (black: 12°C, gray: 16°C) of carbohydrate concentrations. **(A)** apex/source ratio of sucrose; **(B)** apex/source ratio of hexoses; **(C)** sucrose/hexose ratio in the apex. (DoT = days after differentiation of temperature; $n = 12$; data are means ± SE, asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

not possible. However, the concentrations of ABA and IAA in the tissues changed in response to the exposure to sub-optimal temperature. When compared with the control plants, at 3 DoT and 7 DoT the chilled plants contained higher ABA concentrations in the source leaves and in the apex, respectively. ABA concentrations in the apical sink tissue accumulated during the last 2 weeks of the experiment. However, at 28 DoT the ABA concentration in the apex was significantly lower in chilled plants than in control plants (**Figure 4A**). During this period, ABA concentrations neither in the internodes (**Figure 4C**) nor in the source tissue (**Figure 4E**) were affected by temperature. IAA concentrations in the apex were not significant different during the first week (**Figure 4B**). However, during week two and three, concentrations in control plants increased and seemed to reach a steady plateau in week four, whereas concentrations in the chilled plants remained on a significantly lower level, comparable to that at 7 DoT. IAA concentrations in the internodes (**Figure 4D**) were on a generally higher level than in source leaves and increased under chilling treatment continuously from 1 DoT to 21 DoT, without showing the fluctuations, that were observed at 16°C. IAA concentrations in the source tissue (**Figure 4F**) did not vary significantly between 12 and 16°C.

Response of Gene Expression to Mild Chilling

Samples were collected on four dates (1, 3, 7, 21 DoT), to examine fast, midterm and long-term reactions of gene expression to mild chilling temperatures. Even the 4 K reduction of temperature caused significant changes in gene expression. Most M values ($\log_2$ of fold change ratios) for differentially expressed genes were between −1 and −3 for down-regulated or between 1 and 3 for up-regulated putative genes, respectively. However, a few genes showed even M-values below −3 or above 3. **Figure 5** shows the numbers of significantly differentially expressed genes for the different dates and plant tissues. The response patterns were similar for (a) all significantly differentially expressed genes and (f) those with an annotated function. Numbers of differentially expressed genes disclose three phases of stress-reaction, an early phase between 1 and 3 DoT, a midterm phase at 7 DoT, and a late phase at 21 DoT. Furthermore, the chilling response was in general different between the two tissues with more down-regulated genes in the apex (**Figure 5C**) and more up-regulated genes in the source leaf (**Figure 5A**). Interestingly, the response pattern in the internode, which was analyzed only at 21 DoT, was similar to that of the source leaves (**Figure 5B**). The following analysis focuses on the group of genes with annotated functions only.

The transcription of genes in the source tissue (**Figure 5A**) as well as in the shoot apex (**Figure 5C**) responded to chilling within the first day. This fast response was mainly marked by up-regulation in the source leaves (up/down: 22/1) but by down-regulation in the apex (up/down: 4/46). The apex type of response did not change between 1 and 3 DoT (8/46). In source leaves, however, a strong increase in the number of down-regulated genes was observed at 3 DoT reaching the same number as in the apex (46). During the midterm phase (7 DoT), numbers of differentially expressed genes were sharply reduced in both tissues. The decrease in numbers was mainly due to less down-regulated genes in the apex (12) and in the source leaf (5). However, the midterm reaction was followed by a phase of strong regulation of gene expression, so that highest numbers of up- and down-regulated genes were reached at 21 DoT. Then, most up-regulated genes were found in the source leaf (160) compared to down-regulated genes (69) and to the apex (29), while a high number of up-regulated genes (147) was also detected in the internode (**Figure 5B**).

The differentially regulated genes belonged to various functional groups, whose response to mild chilling varied regarding date and plant organ. Numbers of regulated genes in the different groups are illustrated in **Figure 6**, distinguishing between down-regulated genes in the apex at 1 DoT (A) and 21 DoT (B) and up-regulated genes in the source at 21 DoT (C). The groups are identical to or merged of functional classes as indicated in Supplementary Table S1D. At 1 DoT, the functional group "abiotic stimuli" was down-regulated in the apex (**Figure 6A**) without showing any up-regulation in the

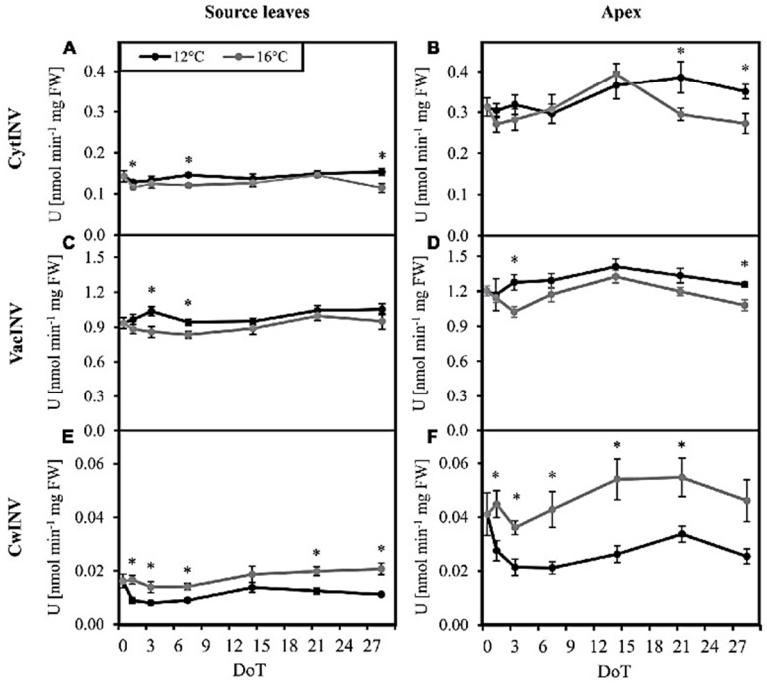


FIGURE 3 | Invertase activities. Impact of sub-optimal temperature (black: 12°C, gray: 16°C) on invertase activities. **(A)** source leaves, cytoplasmic invertase (cytINV); **(B)** apex, cytoplasmic invertase; **(C)** source leaves, vacuolar invertase (vacINV); **(D)** apex, vacuolar invertase; **(E)** source leaves, cell wall invertase (cwINV); **(F)** apex, cell wall invertase. (DoT = days after differentiation of temperature; $n = 10$; data are means ± SE, asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

same tissue or any down-regulation in the source (Supplementary Table S1). The organ-specific response of individual functional groups to the mild chilling became also apparent after longer exposure to the sub-optimal temperatures. At 21 DoT, the functional groups "biotic stimuli" and "ethylene metabolism and perception" were strongly down-regulated in the apex (**Figure 6B**) but not in the source tissue (Supplementary Table S1). However, simultaneously a similar quantity of genes of both groups and the group "chromatin and DNA metabolism" were up-regulated in the source tissue (**Figure 6C**).

Figure 7 considers individual genes and focuses on those, which were differentially expressed at least at two dates, respectively, in two tissues. Supplementary Table S1 shows the full list of all significant differentially regulated genes including their SEQ_IDs. With respect to a particular tissue, most of those genes, which showed regulation at least at two dates, responded in the same direction at different time points. However, only two genes showed consistent expression patterns over all time points analyzed and this was restricted to the source leaves (details discussed below). Comparing the two tissues, contrasting patterns became obvious between the source leaves and the apex. Several individual genes in the functional groups "Abiotic stimuli" and "Biotic stimuli" were down-regulated in the apex, but simultaneously up-regulated in the source leaf and partially in the internode. This applies to some genes of the category "Abiotic Stimuli' at 1 DoT and particularly to genes related to the group "Biotic stimuli" at 21 DoT. Among these were especially genes, which are normally induced by pathogen-induced plant defense responses. Overlaps of genes with the same direction of regulation between source leaf and apex were low in general. However, 49 up-regulated genes overlapped between the source leaves and the internode on 21 DoT, while 28 of these overlapping genes had an annotated protein function.

Also at the level of individual genes, three phases of chilling response became apparent (**Figure 7**). An active early phase during the first 3 days of chilling was marked by the phase-specific and apex-restricted down-regulation of five genes of the category 'Abiotic Stimuli' coding for heat shock proteins and of four genes encoding one Photosystem II subunit, one chalcone synthase G and two auxin-repressed proteins. The subsequent midterm phase after 1 week (7 DoT) with only few genes regulated was followed by a hyper-active gene regulation after 3 weeks (21 DoT). At this time, seven individual genes of the functional group "Ethylene metabolism and perception" were down-regulated in the apex but at the same time up-regulated in the source leaf and/or in the internode (**Figure 7**). These genes were two genes coding for 1-aminocyclopropane-1carboxylate oxidase 4 (SEQ_ID in Supplementary Table S1B: cn2105) and

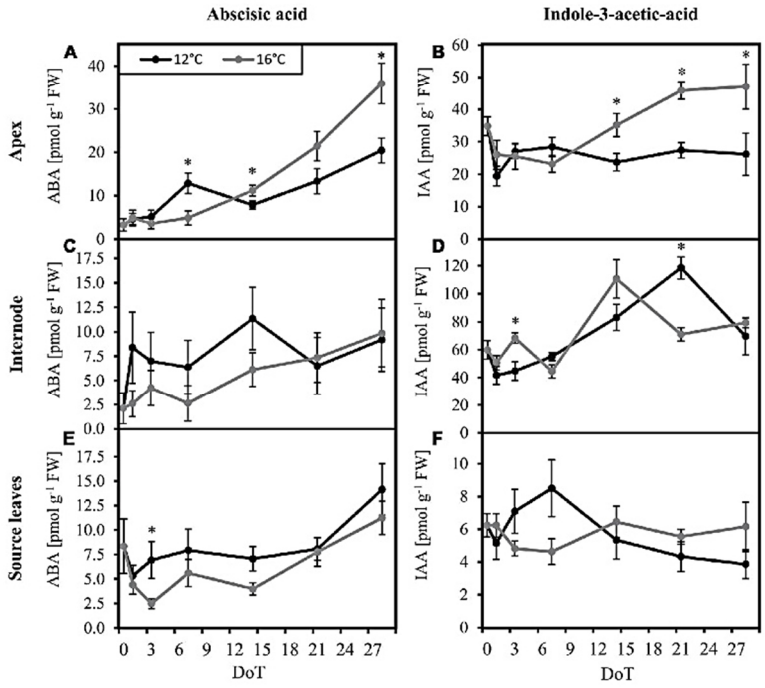


FIGURE 4 | Abscisic acid and indole-3-acetic-acid levels. Impact of sub-optimal temperature (black: 12°C, gray: 16°C) on phytohormone levels. **(A)** apex, abscisic acid (ABA); **(B)** apex, indole-3-acetic-acid (IAA); **(C)** internode, ABA; **(D)** internode, IAA; **(E)** source leaves, ABA; **(F)** source leaves, IAA. (DoT = days after differentiation of temperature; $n = 10$; data are means ± SE; asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

different types of ethylene TFs. Within the category, "Auxin metabolism and perception," one gene coding for a particular auxin-responsive family protein was down-regulated in the late phase in the internode (**Figure 7**). However, no significantly down- or up-regulated genes were found in the functional group of "Abscisic acid metabolism and perception." Looking at "Jasmonate metabolism and perception," two differentially expressed genes were found, both up-regulated in source leaves and the internode at 21 DoT (**Figure 7**). One of them was a gene coding the jasmonate ZIM-domain protein (JAZ, SEQ_ID cn5086), a negative regulator in jasmonate-induced gene expression (Ishiga et al., 2013).

Genes related to different stages of carbohydrate metabolism were regulated under chilling stress. For example, one gene coding for the mitochondrial F1 ATPase subunit, a PS light reaction ATP synthase (GO_drs21P0003D18_R_ab1), was down-regulated in the apex at 1 DoT and in the source at 3 DoT. Three genes coding for AAA-type ATPase were up-regulated at 21 DoT, two in the source leaves (DY395838_1, GO_drs21P0002F21_R_ab1) and one (GO_drs12P0011F24_F_ab1) in the internode. One gene coding for photosystem II subunit R (cn675) was down-regulated in the apex within the early phase (1 and 3 DoT) but in the source and in the internode at 21 DoT. At the same date, down-regulation of four genes coding for RuBisCO activase (cn2051, cn2052, cn2053, cn3274), a type of chaperone that is essential to promote and maintain the catalytic activity of RuBisCO (reviewed in Portis, 2003) further indicated trancriptional inhibition of photosynthesis particularly in the internode. Looking at invertases, three of four cwINV genes were differentially regulated (**Figure 7**; Supplementary Table S1B). In the source leaves, one gene (cn8044) was up-regulated by chilling at 1 DoT and 21 DoT, whereas one other gene (GO_drpoolB-CL9414Contig1) was down-regulated at 21 DoT. However, in the apex chilling repressed the same gene at 3 DoT and two other genes (cn5583, cn8044) at 21 DoT. One gene coding for an invertase inhibitor (cn8301) was down-regulated in the source at 3 DoT whereas one other (GO_drpoolB-CL5724Contig1) was up-regulated in the internode at 21 DoT.

One gene coding for expansin (GO_drpoolB-CL8367Contig1), which is required for leaf growth, was constitutively up-regulated in the source at all dates (1/3/7/21 DoT, **Figure 7**). Also in the source, 17 genes coding for histones H2, H3 and H4 (cn1033, cn1034, cn1037, cn1359, cn1360, cn1361, cn3286, cn4604, cn5095, cn5315, cn5413, cn5414, cn5953, cn8099, cn9045, cn9421, EB174394_1) were up-regulated only at 21 DoT. In addition, two genes coding for chromomethylase (cn9108, GO_drs12P0025N23_F_ab1),

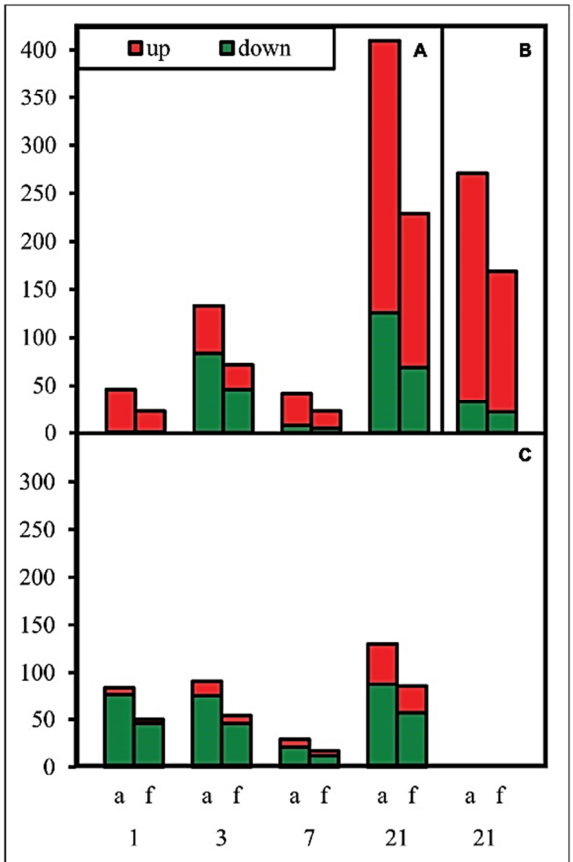


FIGURE 5 | Numbers of differentially expressed genes under chilling stress. Numbers of down- and up-regulated genes (down: chilling < control; up: chilling > control) at different dates after differentiation of temperatures. **(A)** source leaves at 1, 3, 7, 21 DoT; **(B)** internode at 21 DoT; **(C)** apex at 1, 3, 7, 21 DoT. (a, all sequences, f, genes annotated with a known protein function; RankProduct calculated pfp-values <0.15; upregulated: Log_2 >1; down-regulated: Log_2 <−1).

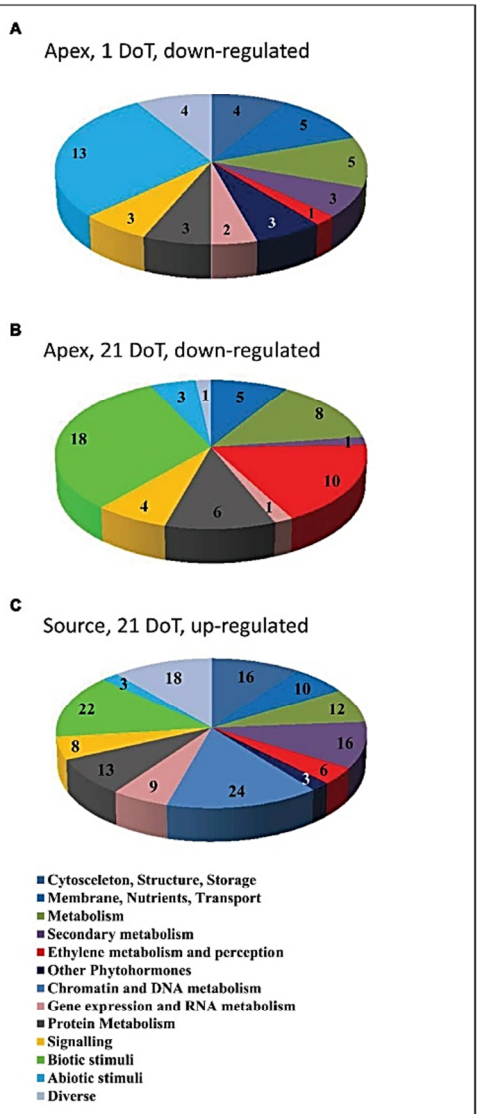


FIGURE 6 | Chilling response of functional groups. Numbers of regulated genes of functional groups (groups see Supplementary Table S1D). Down-regulated in the apex at 1 **(A)** and 21 DoT **(B)** and up-regulated in the source at 21 DoT **(C)**. (Up-regulated: log_2 > 1; down-regulated: log_2 <−1; pfp-value <0.15).

responsible for DNA-methylation (Du et al., 2013b), were up-regulated at 21 DoT. Interestingly, in the apex genes coding for histones did not respond to the chilling stress. **Figure 8** shows differentially expressed TFs that (A) are similar to stress related TFs in *Arabidopsis thaliana* or (B) belong at least to gene-families that are known to include TFs that are involved in stress reactions. A noticeable higher number of differentially expressed TFs, involved in stress signaling, was found in the source tissue and the internode, compared to the apex. TFs showed the strongest reaction to chilling stress at 21 DoT, correlating with the overall higher numbers of differentially expressed genes in the source at 21 DoT, compared to earlier dates and compared to the apex. Differentially regulated genes were found within the TF families of basic helix-loop-helix (bHLH), bZIP family, homeobox, MYB, WRKY, zinc finger family, and others. However, the most continuously affected gene with regard to both, dates and tissues, was a gene coding for the TF Jumonji (jmjC) domain containing protein (GO_dr004P0018C16_F_ab1). It was consistently up-regulated in the source at all dates, in the apex at 3/7 DoT, and in the internode at 21 DoT (**Figure 8**).

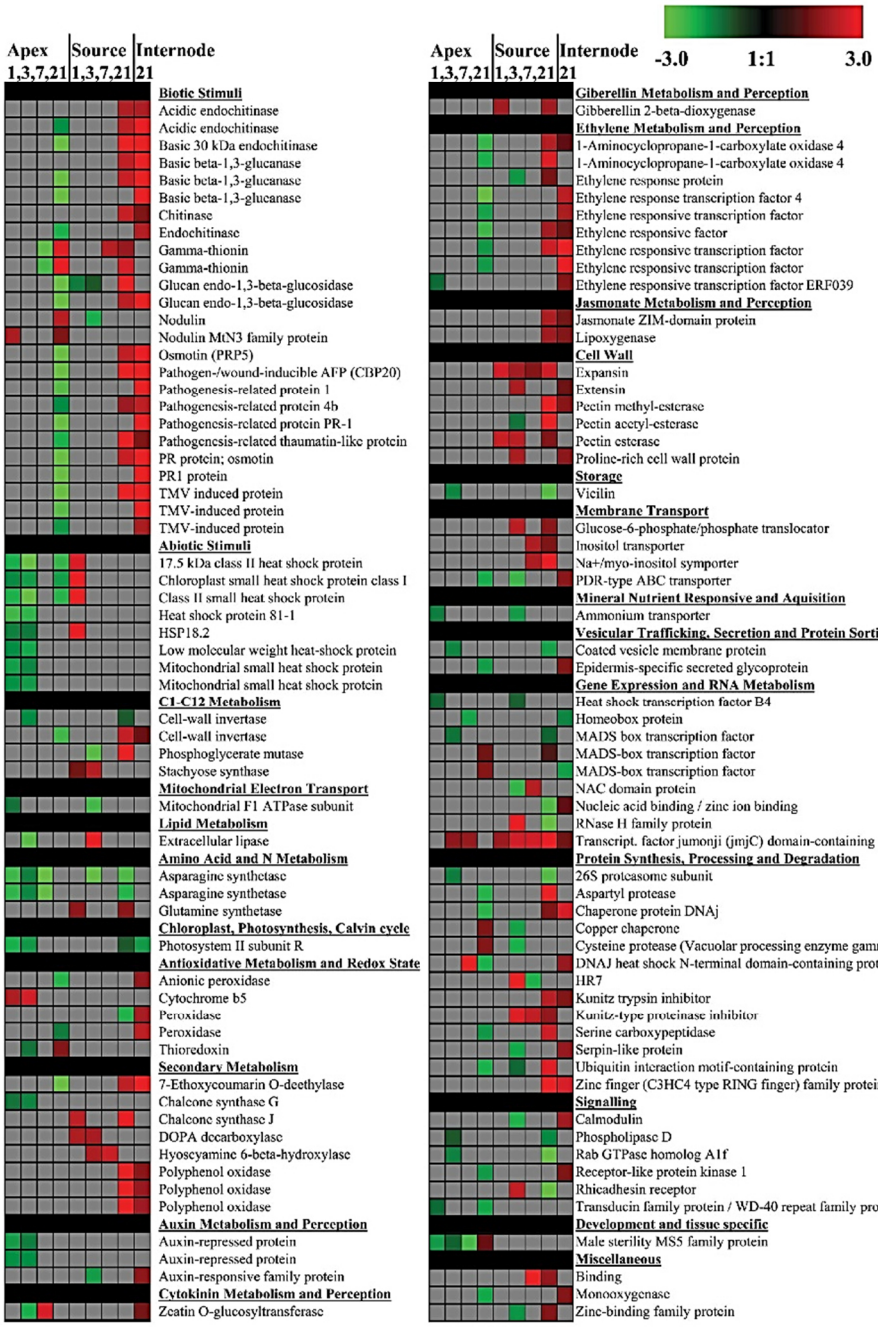
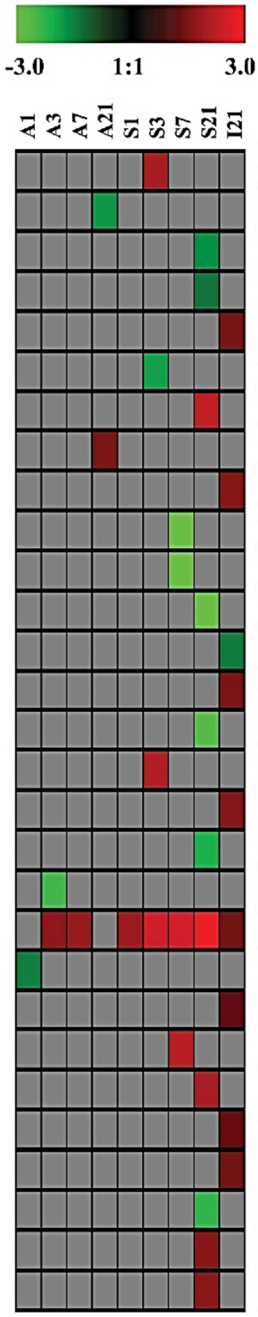


FIGURE 7 | Individual differentially expressed genes. Impact of sub-optimal temperature on expression of individual genes with annotated putative function. Sub-optimal temperature-induced changes are shown for the apex (1, 3, 7, 21 DoT), source leaves (1, 3, 7, 21 DoT), and the internode (21 DoT). The figure focuses on genes, which were differentially expressed at least at two dates or in two organs, respectively. Colored squares represent Log_2 expressions values for individual genes: red color represents up-regulated genes, green color represents down-regulated genes. Intensity of color indicates extend of change in expression (pfp-value < 0.15).



Function	Gene	Similiar to AT gene…	Stress signal involved in: A) Similar AT genes B) Non similar AT genes with similar function
basic helix-loop-helix (bHLH) transcription factor	GO_dr001P0012N09_F_ab1	AT2G43140	**A) COLD**
basic helix-loop-helix (bHLH) transcription factor	cn3038	AT5G50915	B) Several bHLH genes related to COLD and other stress signals
bZIP family transcription factor	cn6623	AT1G35490	B) AT1G27000 related to COLD and other stress signals
bZIP family transcription factor	GO_dr004P0009P21_F_ab1	AT2G40620	
bZIP family transcription factor	cn7218	AT4G34590	**A) ABA, DROUGHT, NACL**
homeobox transcription factor	GO_drpoolB-CL9130Contig1	AT2G46680	**A) DROUGHT, NACL**
homeobox transcription factor	cn9781	AT2G46680	**A) DROUGHT, NACL**
homeobox transcription factor	GO_drpoolB-CL7435Contig1	AT1G69780	B) AT5G44180 related to draught
homeobox transcription factor	IP_PHBS005J12u	AT2G46680	**A) DROUGHT, NACL**
LOB domain-containing protein	cn5718	AT3G02550	B) AT4G37540 related to COLD, NACL
MYB transcription factor	cn2098	AT3G27810	B) Genes related to COLD and other stress signals
MYB transcription factor	cn7717	AT5G57620	
MYB transcription factor	cn7021	AT5G57620	
nam-like protein 3	GI_NP1239993	AT1G01720	**A) ABA, DROUGHT, NACL**
pentatricopeptide repeat-containing protein	GO_dr004P0015E06_F_ab1	AT5G18950	B) Several genes related to COLD and other stress signals
PROTODERMAL FACTOR 1	cn5345	AT2G42840	**A) COLD**
r2r3-myb transcription factor	cn6730	AT4G37260	**A) COLD**
scarecrow transcription factor	IP_PHBS012D08u	AT3G49950	B) AT3G46600 related to COLD and other stress signals
TCP FAMILY TRANSCRIPTION FACTOR	VE_cincinnata	AT3G15030	**A) COLD**
transcription factor jumonji (jmjC) domain-containing protein	GO_dr004P0018C16_F_ab1	AT3G20810	B) AT1G63490 related to COLD, NACL
transcription regulator	GO_dr004P0010L11_F_ab1	AT3G20810	
transcription regulator	cn5933	AT2G46790	**A) COLD**
WRKY transcription factor	GO_dr001P0018I07_F_ab1	AT5G43290	B) Many WRKY transcription factors related to COLD and other stress signals
WRKY transcription factor	SG_SGN-U211100	AT5G56270	
zinc finger (B-box type) family protein	GO_drpoolB-CL3453Contig1	AT1G75540	B) Several genes related to COLD and other stress signals
zinc finger (B-box type) family protein	GO_drs21P0009P06_R_ab1	AT5G48250	
zinc finger (C2H2 type) family transcription factor	GI_NP1239973	AT1G26610	
zinc finger (C2H2 type) family transcription factor	cn1485	AT1G27730	**A) COLD, ABA, DROUGHT, NACL**
zinc finger (C2H2 type) family transcription factor	cn3342	AT3G53600	**A) LIGHT, UV-B**

FIGURE 8 | Transcription Factors. Impact of sub-optimal temperature on expression patterns of transcription factors (TFs) related to TFs that are involved in stress signaling in *Arabidopsis thaliana*. Group **(A)** genes similar to *Arabidopsis* genes coding for TFs related to stress, group **(B)** other genes coding for TFs that belong to TF families in *Arabidopsis* that are related to stress (derived from STIFDB V2.0). Colored squares represent Log_2 expressions values for individual genes: red color represents up-regulated genes, green color represents down-regulated genes. Intensity of color indicates extend of change in expression (pfp-value < 0.15).

Discussion

Exposure of the chilling-sensitive *P. hybrida* cultivar 'Williams' to sub-optimal temperature led to a clear growth depression. The strong reduction of shoot elongation and leaf number contrasting to the absent response of shoot number (Supplementary Figure S2) indicates that sub-optimal temperature impaired growth of individual shoots whereas branching rate by outgrowth of new lateral shoots seems to be less sensitive to moderate chilling. This stays in accordance with similar findings on another *P. hybrida* cultivar reported by Ilias and Rajapakse (2012).

Our findings indicate that the observed growth depression is the outcome of a functional disturbance of the whole plant resulting from a highly dynamic and complex molecular physiological stress response. This is mirrored by changes in carbohydrate metabolism, phytohormone homeostasis, and gene expression patterns in three aerial parts of the plant, which constitute important functional units for plant growth.

Mild Chilling Stress Causes Sugar Accumulation in the Source Leaves at the Long-Term Expense of Carbohydrate Supply to the Apex

Carbohydrate metabolism provides the backbone of plant growth, which is dependent on the availability of carbon sources in the plant and on the transport from source tissues to growing sinks. Furthermore, growth is dependent on carbohydrate utilization at the site of growth. Up to now, only few studies have investigated the response of carbohydrate metabolism to mild chilling temperatures and those studies did not consider different functional units of the plant. Venema et al. (1999) reported increased concentrations of water-soluble carbohydrates and starch accumulation in the youngest mature source leaves of tomato in response to mild chilling temperatures (day/night temperature, control: 20/25°C; chilling: 16/14°C). Nägele et al. (2011) investigated the cold-response (day/night temperature, control: 22/16°C; chilling: 4/4°C) of *Arabidopsis* for a period of 3 days and proposed three consecutive stages of cold acclimation of rosette leaves: an instant dislocation of homeostasis, followed by a restoration of the carbohydrate metabolism and finally a stabilized new homeostasis of carbohydrate metabolism.

To cover the dynamic of both sides of the source-sink network of aerial parts of petunia plants, we monitored carbohydrate levels in the uppermost fully developed leaves as carbohydrate source tissue and in the shoot apex as important utilization sink over a period of 28 days. Even though the microarray data revealed a disturbance of the photosynthetic machinery at transcriptional level, the metabolic data does not reflect a carbohydrate shortage at the source side. By contrast, 'Williams' reacted to exposure to sub-optimal temperatures with a sudden strong increase of hexoses and sucrose already within the first day of treatment, followed by an accumulation of starch from the second day on. While the accumulation of carbohydrates in source leaves was maintained over the whole chilling period of 4 weeks, the increase of carbohydrates in the apex during the first days was followed by intermediate approximation to concentrations of the control between 7 and 14 DoT, and from 21 DoT onward, turned into a reversed situation with lower hexose and starch levels in chilled plants when compared to the controls (**Figure 1**). The carbohydrate ratios between apex and source leaves (**Figure 2**) further highlight the long-term source-sink decoupling under mild chilling stress. While a development-related increase of the apex/source sugar ratios over the time in the control plants indicates an increasing supply of the apex with carbohydrates during the course of plant growth, this change was almost absent under the exposure to chilling temperatures.

Soluble sugars are well known to increase in plant cells at low temperatures and to act there as cryoprotectants that shelter plant cells from freezing (reviewed in Mahajan and Tuteja, 2005). The present carbohydrate data indicates, that the acclimation of the chilling-sensitive petunia cultivar even to the low stress level follows a "defense priority" to protect the source leaves, which have an important maintenance function for the plant. However, sugar accumulation in source tissues competes with carbohydrate export to the sinks. Obviously, under prolonged chilling stress translocation of carbohydrates to the shoot apex and/or their utilization in the apex were reduced and this probably contributed to the growth depression. The observed source-sink decoupling may involve inhibition of carbohydrate export from leaves, of the transport route between the source and sink tissue and also of the sink activity itself, which further drives carbohydrate influx. In this context, the higher sucrose/hexose ratio in the apex of chilled plants from 21 DoT onward indicates a changed sugar equilibrium, which may result from impaired enzymatic sucrose cleavage at the sink side.

Reduction of Activity and Expression of Cell Wall Invertases Particularly in the Apex, but also Other, Post-Transcriptional Processes Seem to be Involved

Since invertases, which are responsible for the turnover of Suc to Fru and Glc and thereby contribute to sink activity (Ahkami et al., 2013), can be affected by low temperature (Usadel et al., 2008), we investigated the activities of cell wall bound (cwInv), cytoplasmic (cytInv), and vacuolar (vacInv) forms of this enzyme. Activities were measured under standard conditions at optimized temperature of 37°C (Ahkami et al., 2013) providing high turnover rates to detect acclimation of invertases to chilling stress. Additional tests at different measuring temperatures (data not shown) showed that the turnover rates per unit time at 16°C, respectively, 12°C were too low for an accurate comparison of enzyme activities at the different current temperatures. Taking into account the general temperature dependency of enzyme activity, the slightly increased levels of vacINV and cytINV activity (**Figures 3A–D**) might even indicate a lower *in vivo* activity at the colder temperatures. Nevertheless, it is an indication for a slight up-regulation of the two invertases particularly in the apex, which might indicate a compensatory response to the above-mentioned insufficient sucrose cleavage. By contrast, the significantly reduced activity of cwINV after exposure of plants to chilling temperatures (**Figures 3E,F**) reflects

an adaptive down-regulation of enzyme activity in response to chilling and is further a strong indication for a chilling-induced reduction of *in vivo* cwINV activity at the lower temperatures, especially in the apex. Considering that correspondingly genes coding for cwINV were found to be down-regulated in the apex, it can be expected that the reduced activity was at least partially the outcome of reduced transcription. Even though the activity of cwINV records only for a one-digit share of total invertase activity, from our results a lower total *in vivo* activity of invertases can be expected at the reduced temperature. Invertases supply hexoses by cleavage of sucrose when there is a high demand for it. Gibeaut et al. (1990) hypothesized from their studies that an osmotic potential gradient caused by an increased invertase activity acts as driving growth factor. Based on this hypothesis, a reduced *in vivo* invertase activity might be one reason for the reduced levels of monosaccharides in the apex in the long-term and the reduced growth of the chilling-sensitive petunia cultivar.

Considering the magnitude of carbohydrate responses to the mild chilling (**Figures 1** and **2**), we assume that also other enzymes are involved in the observed altered carbohydrate homeostasis. Studying cold acclimation in cabbage seedlings, Sasaki et al. (2001) found that enhancement of sucrose synthase and sucrose phosphate synthase activities was related to the increase of hexoses and sucrose. However, in the current gene expression analysis, genes coding for both enzymes were not significantly regulated. In general, the strong response of carbohydrate levels was not associated with a corresponding picture at transcriptional level. While the microarray data reveals a disturbance of the photosynthetic machinery at transcriptional level, it does not explain the carbohydrate accumulation particularly in the source tissue. Therefore, we hypothesize that many of the changes we observed in carbohydrate levels, were influenced by post-transcriptional enzymatic regulations. In addition, enzyme-independent transport of carbohydrates might also be important. Considering that chilling tolerance of maize was correlated to a more efficient export of assimilates from the leaves, Sowiński et al. (2001) stressed that the symplastic route of phloem loading, which is particularly susceptible to low temperature, could play an important role for a high chilling sensitivity.

Mild Chilling Stress Inhibited Long-Term Accumulation of IAA in the Apex, Repressed JA-Signaling, and Stimulated the Ethylene Pathway in an Organ-Specific Manner

Phytohormones are known to be involved in reactions of plants to biotic and abiotic stresses. In rice, JA contents were increased and JA biosynthesis and signaling were induced under cold stress (Du et al., 2013a). In *Arabidopsis*, JA regulates cold acclimation-induced freezing tolerance (Hu et al., 2013). The low JA levels in the present study do not support a role of JA homeostasis in the growth response of the sensitive petunia cultivar to mild chilling stress. However, chilling significantly induced the expression of a jasmonate-ZIM-domain protein, which acts as repressor of jasmonate signaling. Thus, both the missing response of JA levels together with a reduced JA sensitivity may have contributed to the observed growth depression. The results of the presented study further suggest that the homeostasis of IAA and also of ABA are chilling-sensitive factors that may be involved in the growth reaction of petunia to sub-optimal temperatures. According to our results, only transient increases of ABA levels were detected during chilling stress in maize and wheat (Galiba et al., 1993; Anderson et al., 1994; Veisz et al., 1996). However, considering that ABA is obviously involved in acclimation-induced chilling tolerance in maize seedlings (Anderson et al., 1994) and in chilling tolerance of tomato (Ntatsi et al., 2013), the observed lower ABA levels in the growing tissue of shoot apices of petunia plants after prolonged chilling (**Figure 4A**) may be involved in the growth depression. Interestingly, the microarray data does not point toward a regulation of ABA at transcriptional level in the investigated tissues. This may be based on the high proportion of regulated genes without annotated protein function or may indicate that the ABA level in the apex is controlled by biosynthesis in other tissues such as roots (Ntatsi et al., 2013). The transcriptome data does also not provide a clear picture, whether the observed lower IAA level in shoot apices after prolonged exposure to chilling temperatures reflects changed auxin biosynthesis, metabolism, conjugation or transport. Nevertheless, the lower IAA concentrations may have contributed to the growth depression. IAA has a generally important function in the control of cell division, elongation, and growth (Ljung, 2013) and Rahman (2013) emphasized that cold temperatures may strongly change intracellular auxin gradients and reduce intracellular cycling thereby inducing changes in plant growth.

Considering the protective roles of both, ABA biosynthesis and reduced ethylene-signaling in tolerance of tomato against moderately sub-optimal temperature stress, Ntatsi et al. (2013) suggested that the protective role of ABA may be based on indirect control of ethylene action as it was described for conditions of water deficit stress (Sharp et al., 2000; Sharp and Le Noble, 2002). Interestingly, in the present study the expression of several genes related to the group "Ethylene biosynthesis metabolism and signaling" were affected on a long-term by chilling treatment in a tissue-dependent manner (**Figures 6** and **7**). While up-regulation of several genes in the source tissue and the internode indicate a stimulation of ethylene action, most of these genes were simultaneously down-regulated in the apex. Affected by this pattern were genes, that code for aminocyclopropane-1-carboxylate oxidase/∼4 as well as one coding for aminocyclopropane-1-carboxylate synthase, which catalyze the two steps from the precursor *S*-adenosyl-methionine over 1-aminocyclopropane-1-carboxylic acid to ethylene. In *Arabidopsis* seedlings, ethylene biosynthesis was reported to decrease in response to cold stress (Shi et al., 2012). Furthermore, Shi et al. (2012) demonstrated that the tolerance response to freezing stress is negatively regulated by ethylene. Thus, they reduced the freezing tolerance of *Arabidopsis* by inducing an overproduction of ethylene or application of the precursor 1-aminocyclopropane-1-carboxylic acid. Considering these relationships, the chilling-induced growth depression in petunia may also be mediated by ethylene action, possibly in dependence on ABA.

Mild Chilling Stress Caused Phase-Specific and Organ-Specific Changes in Expression of Regulative Components Controlling Stress Response, DNA Replication, and Maintenance

Beyond the changes discussed above, the microarray data reflects a comprehensive phase- and organ-specific transcriptional response of the sensitive *P. hybrida* cultivar 'Williams' to the mild chilling (**Figures 5** and **6**). In accordance to the carbohydrate data, the opposing responses of gene expression in the two tissues during the late phase of chilling may provide indication why growth might have been reduced at the sub-optimal temperatures. On the long term, the plants seemed to attempt to up-regulate stress and DNA metabolism related processes in the source tissue in order to maintain metabolic processes and to adapt to the sub-optimal temperature On the other hand, stress stimuli related genes in the meristematic tissue of the apex and thus, in newly developing leaves were down-regulated. That might have been contributed to saving energy and to channeling resources toward coping with the imposed stress. Interestingly, the functional group "abiotic stimuli" showed a fast response, whereas on the long-term the group "biotic stimuli" was down-regulated. The acute down-regulation of genes related to abiotic stimuli in the developing meristematic tissue probably indicates a higher susceptibility to chilling stress compared with the source tissue. On the other hand, the strong up-regulation of genes related to biotic stimuli in the source leaves and in the internode in the long-term phase, which contrasted to the simultaneous down-regulation of most genes in the apex, might contribute to enhance the tolerance of the source leaves toward the continuing chilling stress. Interestingly, one expansin gene was constitutively up-regulated in the source tissue at all the time points (**Figure 7**). While expansin has primarily a growth promoting function, Goh et al. (2012) supposed its importance for counteracting growth-repressing activities in developed leaves. Considering this hypothesis, expansin seems to act here more as counterbalancing agent against the growth-depressing effects of chilling exposure than as a mere growth promotor.

The transcriptome data reflects a chilling-induced phase- and organ-specific regulation of diverse genes, which control DNA replication and maintenance as well as TFs involved in stress signaling. In the long-term phase, genes coding for different histones, as well as two genes coding for one chromomethylase, similar to chromomethylase 3 from *Arabidopsis*, were over-expressed only in the source tissue. Histones, the core elements of nucleosomes, are *S*-phase dependently expressed, when more DNA is needed. Chromomethylase 3, which is also associated with nucleosomes, has been suggested to methylate nucleosome-bound DNA. Because in *Arabidopsis* chromomethylase 3 is primarily expressed in dividing cells, it is supposed to methylate DNA during replication. However, chromomethylase 3 has also a *de novo* activity on unmethylated DNA, which points toward a maintenance function (Du et al., 2013b). Thus, the over-expression of both, histone genes and chromomethylase genes indicates a demand for more DNA, respectively, regulation of DNA, possibly caused by an induction of cell division or more likely by an induction of repair processes. A Jumonji (JmjC)

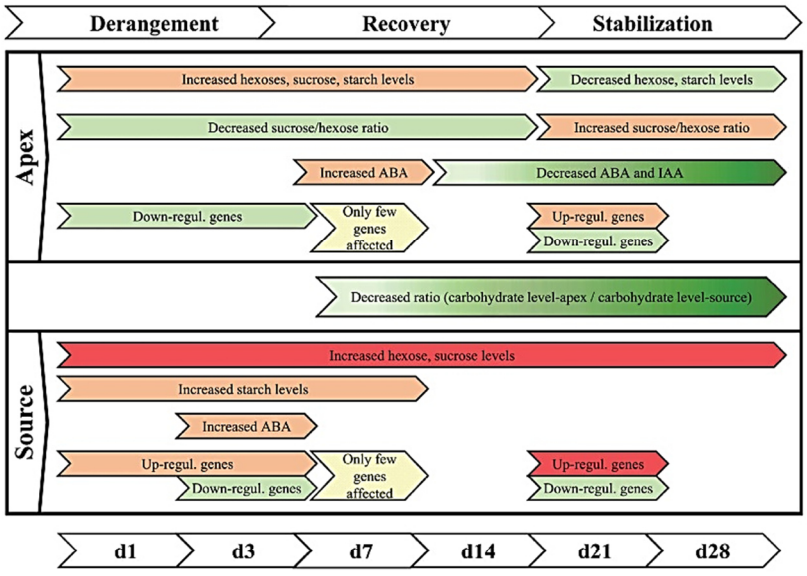


FIGURE 9 | Summary of phases of chilling reaction. The reaction of the sensitive cultivar 'Williams' to the exposure to sub-optimal temperature can be characterized by three consecutive steps of derangement, recovery, and stabilization. In this context, decrease is understood as concentrations that were relative lower compared to control temperature conditions. (Red color indicates increase, green color indicates reduction, while intensity is marked by the intensity of colors; yellow indicates partial alignment to the control plants).

domain-containing protein was one of the most constitutively up-regulated genes. Acting as histone demethylase in *Arabidopsis*, JmjC domain-containing proteins protect euchromatin from heterochromatisation. Thus, these proteins are involved in chromatin remodeling, and they further seem to function as circadian clock component (Lu and Tobin, 2011). According to these functions, the higher expression of JmjC may have contributed to the up-regulation of other genes.

Li et al. (2015) analyzed the cold stress response of cold-tolerant petunia seedlings at low temperature (2°C). They supposed that in petunia, additionally to the CBF-pathway, different other signaling systems are cross-linked to regulate the cold stress response. As potential regulators, they found cold responsive TFs especially of the TF families AP2-EREBP, GRAS, MADS-box, MYB/MYB-related, NAC, and zinc finger (Li et al., 2015). Under our conditions of mild chilling stress, applied to a sensitive petunia cultivar, we also found differentially regulated TFs, especially of the families bHLH, bZIP, ethylene responsive, homeobox, MADS-box, MYB, WRKY, and zinc finger. However, the differential regulation of genes coding for TFs was not stable over the different time points. The above-indicated ICE1–CBF transcriptional cascade has repeatedly been discussed to play an important role in reactions to chilling and cold exposure (Usadel et al., 2008; Zhang et al., 2012; Hu et al., 2013). Interestingly, the results of our microarray analysis did not provide any indication that expression of CBF genes was affected by the mild chilling. On the one hand, there is a chance that some of these genes were not recognized because they are not annotated yet. On the other hand, a lacking induction of CBF family could be related to the chilling sensitivity of the cultivar 'Williams,' since genes of the CBF family are expected to contribute to chilling tolerance. At least the up-regulation of JAZ in the source and the internode at 21 DoT could be an explanation for an absent CBF reaction. Hu et al. (2013) described that the cold induction of CBF/DREB1 signaling pathway itself seems to be up-regulated by jasmonate. JAZ proteins function as repressors of jasmonate signaling and interact physically with the ICE1 and ICE2 TFs. Thus, the transcriptional function of ICE1 is repressed and so the expression of its regulon weakened (Hu et al., 2013). The authors found in *Arabidopsis* that an overexpression of JAZ1 and JAZ4 repressed the freezing stress response. In addition, Zhang et al. (2012) found, by comparing distinct tolerant rice cultivars, a considerably higher recovery capacity of the tolerant cultivar at the transcriptional level.

Conclusion

Even a mild chilling stress, realized with a temperature reduction of only 4 K, leads to a complex disturbance of plant functional integrity of a chilling-sensitive petunia cultivar, detectable at the levels of carbohydrates, phytohormones, and gene expression. The data as a whole reveals a holistic stress response: under chilling, specific functional units of the plant are readjusted and fine-tuned in different directions, so that the whole plant enhances the chance to survive at the expense of growth. Taking in account all presented data, a response model is proposed for a chilling-sensitive cultivar, which comprises three consecutive phases (summarized in **Figure 9**). Immediately after the reduction of temperature, the homeostasis of the plant is deranged, marked by accumulation of hexoses, and up-regulation of genes in the source tissue within the first day and an additional down-regulation at 3 DoT, while in the apex genes are mainly down-regulated on both dates. This phase of destabilization is followed by a transient phase of recovery, characterized by a trend of metabolic values toward a state similar to the plants cultivated at optimal cultivating temperature and a low number of differentially expressed genes. This phase extends into the stabilization phase, which indicates a long-term acclimation to the sub-optimal temperature. It is characterized by lower levels of hexoses, ABA and IAA and a lower sucrose/hexose ratio in the apex and lower apex/source ratios of sugars compared to the control culture. It is further marked by a high number of up-regulated genes particularly of the categories 'Biotic Stimuli' and 'Ethylene metabolism and Perception' in the source tissue, whereas genes of the category 'Biotic Stimuli' are down-regulated in the apex. This late response obviously helps the plant to tolerate the sub-optimal temperature without damage but at the expense of reduced growth. Future experiments will focus on the question, whether cultivars differing in the growth response to mild chilling exhibit tolerance-specific response profiles at the molecular physiological levels described.

Acknowledgments

We thank Sabine Czekalla, Baerbel Broszies, Klaus Fricke, and Kerstin Fischer (all IGZ) for their excellent technical support in cultivating the petunia plants, sampling, performing the plant hormone analysis and extracting the RNA for the microarray studies. We also thank Dr. Siegfried Zerche and Dr. Rita Zrenner for helpful advices regarding the fertilization of plants and analysis of microarray data. We thank Beate ter Hell and Dr. Dirk Ludolph from the Lehr- und Versuchsanstalt für Gartenbau Ahlem for their support in developing the chilling test conditions and Selecta Klemm GmbH & Co. KG (Stuttgart) for recommending the cultivar and providing rooted cuttings. The work was funded by the BMBF (Federal Ministry of Education and Research of the Federal Republic of Germany) within the BMBF-AgroClustEr: WeGa – Horticulture Research Network – Product- und Production safety in intensive Plant production systems and further supported by ministries of the state of Brandenburg, the Free State of Thuringia and the Federal Republic of Germany.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00583

References

Ahkami, A. H., Melzer, M., Ghaffari, M. R., Pollmann, S., Ghorbani Javid, M., Shahinnia, F., et al. (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. doi: 10.1007/s00425-013-1907-z

Ahkami, A., Scholz, U., Steuernagel, B., Strickert, M., Haensch, K. T., Druege, U., et al. (2014). Comprehensive transcriptome analysis unravels the existence of crucial genes regulating primary metabolism during adventitious root formation in *Petunia hybrida*. *PLoS ONE* 9:e100997. doi: 10.1371/journal.pone.0100997

Allen, D. J., and Ort, D. R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends Plant Sci.* 6, 36–42. doi: 10.1016/S1360-1385(00)01808-2

Anderson, M. D., Prasad, T. K., Martin, B. A., and Stewart, C. R. (1994). Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance. *Plant Physiol.* 105, 331–339.

Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19, 185–193. doi: 10.1093/bioinformatics/19.2.185

Breitling, R., Armengaud, P., Amtmann, A., and Herzyk, P. (2004). Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett.* 573, 83–92. doi: 10.1016/j.febslet.2004.07.055

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., et al. (2010). Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. *Plant J.* 64, 1002–1017. doi: 10.1111/j.1365-313X.2010.04385.x

Bundesministerium für Ernährung, Verbraucherschutz und Landwirtschaft. (2013). *Der Gartenbau in Deutschland – Daten und Fakten*. Bonn. Available at: http://berichte.bmelv-statistik.de/GBB-0000100-2012.pdf [accessed 08 April, 2015].

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B., Hong, X., Agarwal, M., et al. (2003). ICE1: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* 17, 1043–1054. doi: 10.1101/gad.1077503

Du, H., Liu, H., and Xiong, L. (2013a). Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Front. Plant Sci.* 4:397. doi: 10.3389/fpls.2013.00397

Du, J., Zhong, X., Bernatavichute, Y. V., Stroud, H., Feng, S., Caro, E., et al. (2013b). Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. 151, 167–180. doi: 10.1016/j.cell.2012.07.034.Dual

Elings, A., Kempkes, F. L. K., Kaarsemaker, R. C., Ruijs, M. N. A., van de Braak, N. J., and Dueck, T. A. (2005). The energy balance and energy - saving measures in greenhouse tomato cultivation. *Acta Hortic.* 691, 67–74.

Galiba, G., Tuberosa, R., Kocsy, G., and Sutka, J. (1993). Involvement of chromosome-5a and chromosome-5D in cold-induced abscisic-acid accumulation in and frost tolerance of wheat calli. *Plant Breed.* 110, 237–242. doi: 10.1111/j.1439-0523.1993.tb00583.x

Gerats, T., and Vandenbussche, M. (2005). A model system for comparative research: *Petunia*. *Trends Plant Sci.* 10, 251–256. doi: 10.1016/j.tplants.2005.03.005

Gibeaut, D. M., Karuppiah, N., Chang, S.-R., Brock, T. G., Vadlamudi, B., Kim, D., et al. (1990). Cell wall and enzyme changes during the graviresponse of the leaf-sheath pulvinus of oat (*Avena sativa*). *Plant Physiol.* 94, 411–416. doi: 10.1104/pp.94.2.411

Goh, H.-H., Sloan, J., Dorca-Fornell, C., and Fleming, A. (2012). Inducible repression of multiple expansin genes leads to growth suppression during leaf development. *Plant Physiol.* 159, 1759–1770. doi: 10.1104/pp.112.200881

Guy, C., Kaplan, F., Kopka, J., Selbig, J., and Hincha, D. K. (2008). Metabolomics of temperature stress. *Physiol. Plant.* 132, 220–235. doi: 10.1111/j.1399-3054.2007.00999.x

Hajirezaei, M., Sonnewald, U., Viola, R., Carlisle, S., Dennis, D., and Stitt, M. (1993). Transgenic potato plants with strongly decreased expression of pyrophosphate:fructose-6-phosphate phosphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. *Planta* 192, 16–30. doi: 10.1007/BF00198688

Hajirezaei, M. R., Takahata, Y., Trethewey, R. N., Willmitzer, L., and Sonnewald, U. (2000). Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. *J. Exp. Bot.* 51, 439–445. doi: 10.1093/jexbot/51.suppl_1.439

Hu, Y., Jiang, L., Wang, F., and Yu, D. (2013). Jasmonate regulates the inducer of cbf expression-C-repeat binding factor/DRE binding factor1 cascade and freezing tolerance in *Arabidopsis*. *Plant Cell* 25, 2907–2924. doi: 10.1105/tpc.113.112631

Hurry, V., Strand, Å., Furbank, R., and Stitt, M. (2000). The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the pho mutants of *Arabidopsis thaliana*. *Plant J.* 24, 383–396. doi: 10.1046/j.1365-313X.2000.00888.x

Ilias, I. F., and Rajapakse, N. (2012). Growth and flowering responses of *Petunia×hybrida* to different photoperiods, light qualities and temperatures. Does light quality affect photosynthetic apparatus of petunia? *Phyt. Ann. Rei. Bot.* 52, 163–175.

Ishiga, Y., Ishiga, T., Uppalapati, S. R., and Mysore, K. S. (2013). Jasmonate ZIM-domain (JAZ) protein regulates host and nonhost pathogen-induced cell death in tomato and *Nicotiana benthamiana*. *PLoS ONE* 8:c75728. doi: 10.1371/journal.pone.0075728

Kim, J., Malladi, A., and van Iersel, M. W. (2012). Physiological and molecular responses to drought in *Petunia*: the importance of stress severity. *J. Exp. Bot.* 63, 6335–6345. doi: 10.1093/jxb/ers285

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. *J. Plant Physiol.* 167, 547–554. doi: 10.1016/j.jplph.2009.11.008

Laing, E., and Smith, C. P. (2010). RankProdIt: a web-interactive Rank Products analysis tool. *BMC Res. Notes* 3:221. doi: 10.1186/1756-0500-3-221

Lee, B., Henderson, D. A., and Zhu, J.-K. (2005). The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell* 17, 3155–3175. doi: 10.1105/tpc.105.035568

Li, B., Ning, L., Zhang, J., Bao, M., and Zhang, W. (2015). Transcriptional profiling of *Petunia* seedlings reveals candidate regulators of the cold stress response. *Front. Plant Sci.* 6:118. doi: 10.3389/fpls.2015.00118

Ljung, K. (2013). Auxin metabolism and homeostasis during plant development. *Development* 140, 943–950. doi: 10.1242/dev.086363

Lu, S. X., and Tobin, E. M. (2011). Chromatin remodeling and the circadian clock: Jumonji C-domain containing proteins. *Plant Signal. Behav.* 6, 810–814. doi: 10.4161/psb.6.6.15171

Mahajan, S., and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.* 444, 139–158. doi: 10.1016/j.abb.2005.10.018

Müller, A., Düchting, P., and Weiler, E. W. (2002). A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to *Arabidopsis thaliana*. *Planta* 216, 44–56. doi: 10.1007/s00425-002-0866-6

Nägele, T., Henkel, S., Hörmiller, I., Sauter, T., Sawodny, O., Ederer, M., et al. (2010). Mathematical modeling of the central carbohydrate metabolism in *Arabidopsis* reveals a substantial regulatory influence of vacuolar invertase on whole plant carbon metabolism. *Plant Physiol.* 153, 260–272. doi: 10.1104/pp.110.154443

Nägele, T., Kandel, B. A., Frana, S., Meissner, M., and Heyer, A. G. (2011). A systems biology approach for the analysis of carbohydrate dynamics during acclimation to low temperature in *Arabidopsis thaliana*. *FEBS J.* 278, 506–518. doi: 10.1111/j.1742-4658.2010.07971.x

Naika, M., Shameer, K., Mathew, O. K., Gowda, R., and Sowdhamini, R. (2013). STIFDB2: an updated version of plant stress-responsive transcription factor database with additional stress signals, stress-responsive transcription factor binding sites and stress-responsive genes in *Arabidopsis* and rice. *Plant Cell Physiol.* 54, 1–15. doi: 10.1093/pcp/pcs185

Ntatsi, G., Savvas, D., Druege, U., and Schwarz, D. (2013). Contribution of phytohormones in alleviating the impact of sub-optimal temperature stress on grafted tomato. *Sci. Hortic.* 149, 28–38. doi: 10.1016/j.scienta.2012.09.002

Portis, A. R. (2003). Rubisco activase - Rubisco's catalytic chaperone. *Photosynth. Res.* 75, 11–27. doi: 10.1023/A:1022458108678

Rahman, A. (2013). Auxin: a regulator of cold stress response. *Physiol. Plant.* 147, 28–35. doi: 10.1111/j.1399-3054.2012.01617.x

Rasmussen, A., Hosseini, S. A., Hajirezaei, M., Druege, U., and Geelen, D. (2015). Adventitious rooting declines with the vegetative to reproductive switch and involves a changed auxin homeostasis. *J. Exp. Bot.* 66, 1437–1452. doi: 10.1093/jxb/eru499

Sasaki, H., Ichimura, K., Imada, S., and Yamaki, S. (2001). Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. *J. Plant Physiol.* 158, 847–852. doi: 10.1078/0176-1617-1

Schwarz-Sommer, Z., Davies, B., and Hudson, A. (2003). An everlasting pioneer: the story of *Antirrhinum* research. *Nat. Rev. Genet.* 4, 657–666. doi: 10.1038/nrg1127

Sharp, R. E., and Le Noble, M. E. (2002). ABA, ethylene and the control of shoot and root growth under water stress. *J. Exp. Bot.* 53, 33–37. doi: 10.1093/jexbot/53.366.33

Sharp, R. E., Le Noble, M. E., Else, M. A., Thorne, E. T., and Gherardi, F. (2000). Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene. *J. Exp. Bot.* 51, 1743–1751. doi: 10.1093/jexbot/51.350.1575

Shi, Y., Tian, S., Hou, L., Huang, X., Zhang, X., Guo, H., et al. (2012). Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in *Arabidopsis*. *Plant Cell* 24, 2578–2595. doi: 10.1105/tpc.112.098640

Shibasaki, K., Uemura, M., Tsurumi, S., and Rahman, A. (2009). Auxin response in *Arabidopsis* under cold stress: underlying molecular mechanisms. *Plant Cell* 21, 3823–3838. doi: 10.1105/tpc.109.069906

Sowiński, P., Rudzińska-Langwald, A., Dalbiak, A., and Sowińska, A. (2001). Assimilate export from leaves of chilling-treated seedlings of maize. The path to vein. *Plant Physiol. Biochem.* 39, 881–889. doi: 10.1016/S0981-9428(01)01302-X

Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002). Genesis: cluster analysis of microarray data. *Bioinformatics* 18, 207–208. doi: 10.1093/bioinformatics/18.1.207

Usadel, B., Bläsing, O. E., Gibon, Y., Poree, F., Höhne, M., Günter, M., et al. (2008). Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. *Plant. Cell Environ.* 31, 518–547. doi: 10.1111/j.1365-3040.2007.01763.x

Veisz, O., Galiba, G., and Sutka, J. (1996). Effect of abscisic acid on the cold hardiness of wheat seedlings. *J. Plant Physiol.* 149, 439–443. doi: 10.1016/S0176-1617(96)80146-5

Venema, J. H., Posthumus, F., and van Hasselt, P. R. (1999). Impact of suboptimal temperature on growth, photosynthesis, leaf pigments and carbohydrates of domestic and high-altitude wild *Lycopersicon* species. *J. Plant Physiol.* 155, 711–718. doi: 10.1016/S0176-1617(99)80087-X

Walworth, A. E., Song, G., and Warner, R. M. (2014). Ectopic AtCBF3 expression improves freezing tolerance and promotes compact growth habit in petunia. *Mol. Breed.* 33, 731–741. doi: 10.1007/s11032-013-9989-7

Warner, R. M. (2010). Temperature and photoperiod influence flowering and morphology of four *Petunia* sp. *HortScience* 45, 365–368.

Yamori, W., Noguchi, K., Hikosaka, K., and Terashima, I. (2009). Cold-tolerant crop species have greater temperature homeostasis of leaf respiration and photosynthesis than cold-sensitive species. *Plant Cell Physiol.* 50, 203–215. doi: 10.1093/pcp/pcn189

Zhang, T., Zhao, X., Wang, W., Pan, Y., Huang, L., Liu, X., et al. (2012). Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes. *PLoS ONE* 7:e43274. doi: 10.1371/journal.pone.0043274

Zhu, J., Dong, C.-H., and Zhu, J.-K. (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.* 10, 290–295. doi: 10.1016/j.pbi.2007.04.010

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.3 Comparative analysis of two contrasting petunia cultivars indicates important functions of carbohydrate utilization and abscisic acid in tolerance to mild chilling stress

Comparative analysis of two contrasting petunia cultivars indicates important functions of carbohydrate utilization and abscisic acid in tolerance to mild chilling stress

Martin Andreas Bauerfeind[1], Susanne Amberger-Ochsenbauer[2], Philipp Franken[1], Uwe Druege[1]*

[1]Department Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Erfurt, Germany

[2]Weihenstephan-Triesdorf University of Applied Sciences, Freising, Germany

*** Correspondence:** Uwe Druege, Department Plant Propagation, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Kuehnhaeuser Str. 101, 99090 Erfurt, Germany, e-mail: druege@erfurt.igzev.de

Abstract

Background: Energy savings in production of thermophilic ornamental crops could be realized with tolerant cultivars that show minimal growth depression in response to sub-optimal temperature. To identify factors that might control chilling tolerance of *Petunia hybrida*, the chilling-tolerant cultivar (cv) `Ultra Blue´ (UB) was compared with the sensitive cv `SweetSunshine Williams´ (SW). We investigated dynamics of carbohydrate metabolism by enzymatic assays, photosynthetic activity by gas exchange measurement, homeostasis of phytohormones by GC-MS/MS and gene expression by a petunia specific microarray over a chilling period (12 °C versus 16 °C) of three weeks.

Results: UB was characterized by a lower chilling-induced increase of soluble sugars in the source organs but constitutively higher sucrose levels in the apex and higher net photosynthesis at both temperatures. Furthermore, activities of cytosolic and cell wall invertase were constitutively higher for UB than for SW particularly in the apex and were associated with lower expression of genes coding for invertase inhibitors in the different organs. In UB one gene coding for phosphoenolpyruvate carboxylase kinase was higher expressed in the apex. Abscisic acid (ABA) displayed three fold higher concentrations in UB at both temperatures, especially in the apex. This

was associated with generally higher hybridization signals for a gene encoding a carotenoid cleavage dioxygenase independent of the temperature. Application of ABA to SW enhanced ABA levels and reduced biomass production at both temperatures, whereas treatment of UB with nordihydroguaiaretic acid (NDGA), an inhibitor of ABA-biosynthesis, reduced ABA levels but enhanced biomass production only at control temperature. ABA treatment reduced the chilling-induced growth depression and enhanced leaf development in chilled SW, whereas NDGA treatment enhanced the chilling-induced growth depression.

Conclusions: Metabolic and transcriptome data suggest that higher and more stable carbohydrate transport to and carbohydrate turnover in the growth sinks contribute to higher tolerance to mild chilling in petunia. The results further support a protective role of ABA, which might depend on the intrinsic distribution within the plant. Ethylene-, auxin-, and gibberellin-related pathways seem also to be involved. Functional analysis of candidate genes will contribute to a better understanding of the processes controlling tolerance to mild chilling stress.

Keywords: cold, gene expression, invertase, low temperature, microarray, *Petunia hybrida*, photosynthesis, plant hormones, SAUR

Background

Heating of greenhouses is a significant cost factor for the production of thermophilic ornamental crops during the winter months in northern climates. In respect to the rising energy costs as well as to the growing environmental awareness of customers, energy saving is becoming more and more important as integral part of sustainable crop production. Producing at lower temperatures would reduce energy inputs but also impose chilling stress on the plants causing damage or at least extend the time of production, which would counteract the energy saving goals. To realize an efficient sustainable production of thermophilic plant species, cultivars (cvs) are needed that are chilling-tolerant, which means that they are only slightly affected in their growth when exposed to sub-optimal temperatures. It is known for many species, such as *Petunia hybrida*, that chilling susceptibility varies between cvs.

Petunia belongs to the top five bedding plants in Germany with a market volume of more than € 100 million in 2012 [1]. Nevertheless, processes on molecular level underlying variations in chilling tolerance of petunia have not been adequately investigated as of many other ornamental crops. Whereas detailed analyses of contrasting chilling-tolerant and -sensitive cvs on mild chilling conditions already exist for several crop species, e.g. for rice (10 °C vs. 25 °C) [2] and vegetables like spinach (15 °C vs. 30 °C) [3], for petunia such comparative molecular physiological investigations are lacking. A detailed knowledge of the molecular regulation of chilling tolerance in petunia in a range of temperature, practically relevant to production, would help breeders to enable a more efficient

breeding technology towards lower energy demand of petunia for example via functional markers and thus promote more sustainable crop production.

The chilling response is a complex interplay of reactions that affect the integrity of the whole plant metabolism. The carbohydrate metabolism including photosynthesis and the homeostasis of stress-related phytohormones are involved, and these multifaceted responses are regulated on gene expression level. Considering low temperature stress, the question arises whether responses and tolerance to sub-optimal production temperatures such as 4 - 6 K below the usual production temperature involve the same physiological principles when compared to stronger cold stress at temperatures close to or even below the freezing point. In rosette leaves of *Arabidopsis*, changes in plant metabolism and gene expression were already observed at slightly decreased ambient temperatures and were qualitatively not different to the changes induced by lower temperatures, while the responses to chilling increased gradually with decreasing temperatures [4]. With increasing severity of chilling (from 20 °C to 17, 14, 12, 10 or 8 °C), levels of carbohydrates and many other stress-responsive metabolites progressively increased in the cells [4]. Some of these metabolites like soluble sugars shelter cells from freezing [5]. However, increased levels of sugars and starch were observed in young mature source leaves of tomato already at a moderate reduction of temperature (16/14 °C vs. 25/20 °C) [6], when cryoprotection was not essential for survival. Sucrose and the hexoses fructose and glucose are beyond their energy, organic carbon and osmotic functions important signal molecules regulating source-sink relations in general and particular under stress [7]. Therefore, altered carbohydrate levels can be expected both, to be the result of a modified source-sink-regulation, but also to affect directly the source-sink crosstalk. In contrast to the increasing carbohydrate levels, photosynthetic rates have been frequently observed to decrease at reduced temperatures [8]. However, when low light levels impair gross photosynthesis, decrease in temperature below 20 °C can enhance net photosynthetic rate, which may result from reduced respiration [9]. Yamori et al. [10] investigated the plasticity of photosynthetic temperature acclimation in different crop species. They found, that cold-tolerant plants featured a higher flexibility in various photosynthetic parameters during cold acclimation, and by that they were able to tolerate bigger decreases in optimum temperature for the photosynthetic rate (CO_2 concentration: 360 µL L^{-1}) [10]. Thus, the cold tolerance of the investigated species seemed to be based on a greater temperature homeostasis of photosynthesis and leaf respiration over a bigger extent of temperature changes than in cold-sensitive plants [11].

As key enzymes for the hydrolysis of sucrose into fructose and glucose, invertases play a crucial role in carbohydrate partitioning. Especially extracellular cell wall invertases (cwINV) are crucial for an apoplastic phloem-unloading pathway and therefore of importance for the carbohydrate translocation and the supply of sink organs. An up-regulation of extracellular invertases seems to be part of the

stress-related reactions to several abiotic and biotic stimuli [12] and that might be, at least partly, due to an altered post-translational regulation. Thus, small proteins act as invertase inhibitors that form inactive complexes with the invertases and by that silence the invertase activity [13,14]. In mature healthy *Arabidopsis* leaves, invertase inhibitors keep the invertase activity repressed. During stress-related stimuli like pathogen attack or wounding, a repression of inhibitor activity increases the activity of cell wall-bound invertases [15]. The authors suggest that this post-transcriptional de-repression of the invertase activity supports the source leaf to establish a localized sink metabolism to enhance the energy supply, needed for the activation of defense reaction cascades.

Phytohormones are involved in plant reactions to chilling. In tomato, mild chilling tolerance could be related to ABA biosynthesis, even if no accumulation of ABA seemed to be necessary to alleviate the depressing effect of chilling on the shoot growth [16]. In maize seedlings, Anderson et al. [17] found that enhanced ABA synthesis, accompanied by an intense rise in free ABA levels, was essential for acclimation-induced chilling tolerance that enables a higher survival rate of seedlings at low temperatures (5 °C). Furthermore, exogenous ABA application onto leaves of *Trichosanthes kirilowii* seedlings decreased malondialdehyde levels, which indicates an increased chilling tolerance under cold environments (4 °C) [18]. Due to its important regulatory role in the control of cell division, elongation and specification, auxin, mainly indole-3-acetic acid (IAA) is crucial for the general regulation of plant growth and development. Considering the response of auxin homeostasis to environmental changes including low temperature, Rahman [19] suggested that reduced development and growth in response to sub-optimal temperature might be linked to altered local auxin gradients. Jasmonic acid (JA) may be involved in the chilling response in rice, too [20]. At freezing temperatures, endogenous JA production was enhanced in *Arabidopsis*, and JA application stimulated the cold induction of genes acting in the CBF/DREB1 signaling pathway (CBFs: C-repeat binding factors; DREB: dehydration-responsive-element-binding protein) that is mediated by CBF/DREB1 proteins, a family of transcription factors, which seem to be crucial for the cold-response pathways in *Arabidopsis* [21,22]. The expression of members of this transcription factor family is triggered by cold, while the transcription factors in turn, increase plant freezing tolerance by controlling the transcription of a regulon of cold-induced genes [23,24]. Usadel et al. [4] supposed that in *Arabidopsis* the CBFs have a critical role already for the response of gene expression and the adaption of the metabolism to mild chilling temperature. Walworth et al. [25] proved the existence of a functional CBF cold-response pathway in petunia and its importance for enhancing freezing tolerance. Nevertheless, beside the CBF pathway, an interplay of different other signaling systems seems to control the cold stress response in petunia [26,27].

In a previous study, we described three consecutive phases of the molecular physiological response of the sensitive cv `SweetSunshine Williams´ (SW) to mild chilling provoked by decrease in temperature

from 16 °C (day/night: 17/15 °C) to 12 °C (13/11 °C), which caused a significant growth depression without any visible cold damage. Carbohydrate levels, phytohormones and gene expression in different organs reflected an early derangement of plants during the first days of mild chilling, followed by a partial recovery after one week and a stabilization phase later on. In that work, we did not find any evidence that the CBF pathway was already involved under the influence of the mild chilling [26]. Based on these results, in the present study we conducted a comparative analysis of the chilling-tolerant cv UB against the sensitive cv SW to elucidate putative candidate factors and processes relevant for tolerance to mild chilling in *P. hybrida*. UB shows a less strong growth depression by reduction of temperature from 16 °C to 12 °C when compared to SW. To cover the functional system of the aerial part of the plants, we considered young but fully developed leaves, the upper stem and the shoot apex as important functional units representing the carbohydrate source, the elongating transport route and the main utilization sink of the growing shoot. We followed the hypothesis that the differences in growth reaction to sub-optimal temperature are related to divergent changes in the carbohydrate metabolism, phytohormone homeostasis, and/or gene expression, of the two cvs. Therefore, we analyzed carbohydrate concentrations and invertase activities by enzymatic assays, determined photosynthetic activities by gas exchange measurement and measured the concentration of the phytohormones ABA, IAA and JA by gas chromatography-tandem mass spectrometry (GC-MS/MS). Moreover, we used a specific petunia microarray, which provides 24,816 unique, non-redundant annotated sequences [28,29] to monitor responses of the transcriptome.

Methods

Plant material and chilling treatment

For the comparison of distinct chilling-sensitive *P. hybrida* cvs, the chilling-sensitive cv `SweetSunshine Williams´ (SW) and the chilling-tolerant `Ultra Blue´ (UB) were chosen. For chilling treatment, an average day temperature of 12 °C was applied and compared to a control of 16 °C average day temperature. The latter is commonly used in German greenhouse production of petunia. This reduction in temperature of 4 K already caused a delayed growth in both cvs, but to different degrees, both without any visible cold damage. Young plants were established from cuttings as described by Bauerfeind et al. [26]. All experiments were conducted in climate chambers. For acclimation to the control conditions (day/night temperatures: 17 °C/15 °C; relative humidity: 70%) and to establish a good rooting system, rooted cuttings were transferred into a climate chamber immediately after potting. The photoperiod lasted 12 h per day (photosynthetic photon flux density: during the first week 100 µmol m^{-2} s^{-1}, from the second week onwards 150 µmol m^{-2} s^{-1}; fluorescent tubes, FQ80W/865 HO Constant, Lumilux Cool Daylight, Osram, München, Germany). For chilling treatment, half of the plants were relocated to a climate chamber with identical conditions, but exposed to chilling temperatures (day/night: 13 °C/11 °C) after two weeks. Because investigations during the four-week period of temperature treatment focused on the vegetative growth of plants,

developing flower buds were constantly removed as soon as visible to prevent interference with competing sinks.

Growth analysis and collection of samples

For determination of dry and fresh weight production, fresh weights of the aerial parts of 12 plants per treatment were measured after three weeks of chilling treatment. Dry weights were determined after drying at 80 °C for 48 h until a constant weight was reached. For detailed evaluation of growth, the elongation of the main shoot was measured and the numbers of newly developed lateral shoots and leaves of the main shoot (marked at the start of differentiation of temperature) were evaluated after three weeks of chilling treatment.

Collection time during the day, sampling of source leaves and of shoot apex, and the treatment and storage of samples was conducted according to Bauerfeind et al. [26]. Additionally, the uppermost stem (1.5 cm, leaves removed), located directly below the harvested apex, was collected for phytohormone and gene expression analyses. The samples for the analyses of carbohydrates, enzymes and phytohormones were collected at 0, 1, 3, 7, 21 days after differentiation of temperature (DoT), the samples for the microarray analysis were collected at 3, 7, and 21 DoT.

Analysis of carbohydrates, invertase activities and phytohormones

Fructose, glucose and sucrose were analyzed using enzymatic assays as described by Hajirezaei et al. [30] and Klopotek et al. [31]. Activity measurements of invertases were conducted as described by Hajirezaei et al. [30,32]. Per each treatment and date, nine biological replicates were analyzed. Extraction, purification and analysis of the phytohormones ABA, IAA and JA by GC-MS/MS were performed as described by Ahkami et al. [33]. One ml methanol containing 10.3 pmol $(^2H)_2$-IAA, 10.3 pmol $(^2H)_6$-ABA and 27.8 pmol $(^2H)_6$-JA as internal standards was added to frozen samples before extraction. Gas chromatography and mass spectrometry settings for ABA, IAA and JA were applied as described by Ahkami et al. [33], Ntatsi et al. [16] and Rasmussen et al. [34]. Per each treatment and date, six biological replicates were analyzed.

Measurement of net photosynthetic rate

An independent experiment was conducted for measuring net photosynthetic rate. Conditions of plant cultivation were identical as described above with the difference that illumination was provided with fluorescent tubes of the type Lumilux Cool White 840 (Osram, München, Germany) and the differentiation of temperature started three weeks after potting of the plants. While plants were cultivated in greenhouse cabins, the actually realized temperatures were 19/15 °C (control, day/night) and 14/11 °C (chilling, day/night). The measurements of gas exchange rates were performed at 1, 3, 7 and 14 DoT. To enable to perform measurements referring to one specific DoT on two consecutive

days, for half of the chilling treated plants the first day of chilling treatment was postponed by one day. For each cv and DoT, two of the four replications were measured in the morning and two in the afternoon, respectively. To eliminate root respiration, the pots of the plants were placed in a polyethylene bag and closed at the stem basis by an elastic sealing. The gas exchange rate of the whole plant was measured in an open system (cuvette volume 15 L) with approximately 400 ppm CO_2 and calculated per leaf dry weight. The relative humidity was adjusted to 70 % by a bypass system and resulted in a saturation deficit of 5.8 hPa for the control and 4.5 hPa for the chilling treatment. Irradiation was applied by metal halide lamps (photosynthetic photon flux density: 150 µmol m^{-2} s^{-1}; Osram Power Star HQI-T, 400 W). The volumetric flow rate was adjusted to 3 ± 1 L min^{-1} and the air circulation to 0.2 – 0.3 m s^{-1}. The steady-state CO_2 uptake rates were determined using infrared gas analysis (Binos 100, Fisher-Rosemount GmbH & Co., Hasselroth, Germany) 45 min after the installation of the plants in the cuvette.

Microarray hybridization and statistical analysis

For gene expression analysis, a petunia-specific microarray was used, which was first described by Breuillin et al. [29]. It carries 24,816 unigene annotated sequences [35]. RNA from three replicates (each replicate was a pooled sample of four individual plants) was extracted with the QIAGEN RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Following the Qiagen protocol, RNA was treated with DNase for extraction. The array hybridization was performed by OakLabs GmbH (Hennigsdorf, Germany) with a minimum of 500 ng total RNA per sample. Normalization of the data was conducted by OakLabs using the Quantil-normalization according to Bolstad et al. [36]. A Rank Product online-analysis (http://strep-microarray.sbs.surrey.ac.uk/RankProducts/) was carried out to identify statistically significant chilling responses and differences between the cvs, respectively [37,38]. Therefore, the expression values of each three replicates grown at sub-optimal temperature (12 °C) were compared with the corresponding samples from the same cv grown at control temperature (16 °C) for each date (3, 7 and 21 DoT). Analogous, hybridization signals of genes in UB were compared with hybridization signals in SW separately for both temperatures to find constitutive or chilling-specific different RNA accumulation values between the contrasting cvs. To indicate the intensity of up- or down-regulation of genes under exposure to chilling, respectively the ratio of hybridization signals of both cvs was calculated and transferred to M-values (Log_2 of ratios). Thus, $Log_2 < -1$ was defined as down-regulated/lower hybridization signal, $Log_2 > 1$ as up-regulated/higher hybridization signal. To identify statistically significant differentially expressed genes, a pfp (probability of false prediction) value threshold of < 0.15 was applied. Expression graphs were created with Genesis, software version 1.7.6 [39].

Spray treatment with abscisic acid and nordihydroguaiaretic acid

Plants for the treatment with (+) abscisic acid (ABA, NBS Biologicals Ltd., Huntingdon, UK) respectively with the ABA-synthesis inhibitor nordihydroguaiaretic acid (NDGA, Sigma-Aldrich, St. Louis, USA) were cultivated as described above. SW was treated with ABA (0, 30, 120, 480 mg/L equivalent to 0, 0.11, 0.45, 1.82 mM), UB was treated with NDGA (0, 15.1, 30.2, 60.5 mg/L equivalent to 0, 0.05, 0.10, 0.20 mM). The NDGA stock solutions were prepared with concentrated ethanol, the ABA stock solutions with H_2O containing 30% ethanol. To reach final concentrations, solutions were diluted with H_2O and adjusted to a final ethanol concentration of 0.4%. Tween (200 µl/L) was added as wetting agent to assure a complete wetting of the treated plants. Plants were sprayed with ABA respectively NDGA solution until complete moistening, while in both treatments 0 mM was used each as a control. The first treatment was applied two days before differentiation of temperature (-2 DoT) and then repeated at weekly intervals (7, 14, 21 DoT). Dry and fresh weights as well as the growth parameters, number of shoots, length of the main shoot, and the leaves on the main shoot, were recorded on -1 DoT and one week after the last spray treatment (28 DoT). In addition, apex samples for phytohormone analyses were taken at 28 DoT.

Statistics of data related to growth, metabolism and phytohormones

The STATISTICA software package (StatSoft, Inc. [2014]. STATISTICA for Windows [data analysis software system], version 12.0. www.statsoft.com) was used for statistical analyses. ANOVA was used in combination with Tukey HSD test to detect differences between treatments as long as variance homogeneity and normal distribution within the groups were met. Variance homogeneity was tested by the Levene's test and normal distribution by the Kolmogorov-Smirnov test. A non-parametric test for comparing means (Mann-Whitney-U test or Kruskal-Wallis test) was applied and tested for significance, if these assumptions were not fulfilled [33]. Significant differences were marked in the respective figures by asterisks (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.01$).

Results

Growth as affected by cv and mild chilling

Both cvs responded with decreases in fresh weight (FW) and dry (DW) production to exposure to chilling. Production of FW (Additional file 1: Figure S1 A) and DW (Additional file 1: Figure S1 B) during the three weeks of temperature treatment differed not significantly between both cvs at control temperature. Contrastingly, the production of FW and DW was significantly higher for UB compared with SW under the influence of chilling, reflecting a reduced growth depression by chilling. Weight productions of UB were reduced by 40% in FW (absolute depression of 12.2 g) and 27% in DW (absolute depression of 0.6 g), whereas the productions of SW were significantly stronger reduced by 57% in FW (absolute depression of 17.7 g) and 47% in DW (absolute depression of 0.9 g) (Additional file 1: Figure S1 C - F). The elongation of the main shoot as well as the production of new leaves and

lateral shoots on the main shoot was reduced under chilling. However, only the length of the main shoot and the development of lateral shoots were stronger affected in SW (Additional file 2: Figure S2).

Carbohydrate levels and ratios as affected by cv and mild chilling

Considering the important role of sugars, especially of sucrose (Suc) and the hexoses fructose (Fru) and glucose (Glc) in source-sink regulation particularly under the influence of stress factors, we assumed tolerance-specific reaction patterns of the carbohydrate levels to the chilling temperature. The source and sink organs of both cvs showed general cultivar-specific differences and a strong response to chilling while the magnitude and dynamics of the chilling reaction of the sugars particularly of sucrose was dependent on the cultivar (Significance Table in Fig. 1). In the source organ, the concentrations of hexoses (Fig. 1 A), Suc (Fig. 1 C) and starch (Fig. 1 E) varied only marginally between both cvs at 16 °C, whereas under chilling treatment, distinct reaction patterns were observed. Hexoses, Suc and starch levels increased immediately after the exposure to chilling. At the same time, sugar levels in the sensitive cv SW responded much stronger (Fig. 1 A). Whereas the tolerant cv UB reached a hexose peak already at 1 DoT of chilling treatment and showed a subsequent decline towards control concentrations thereafter, hexose levels in SW accumulated until 7 DoT when levels were threefold higher compared with UB. The Suc levels in leaves showed an even stronger increase in SW reaching much higher concentrations than in UB at 7 DoT. While high concentrations were maintained in SW until 21 DoT, concentrations increased in UB, up to a level still below that of SW (Fig. 1 C). Additionally, starch levels simultaneously increased as reaction to chilling in both cvs until 3 DoT, followed by a decrease to levels similar to control plants (Fig. 1 E), with similar levels recorded for both cultivars.

A significant cultivar effect was also found in the apex, at 16 °C, especially for Suc. However, larger differences were detected between cvs at 12 °C at least for sucrose and starch. Levels of hexoses were slightly higher in UB at 0, 3 and 7 DoT at both temperatures. A transient chilling-mediated increase in hexose levels observed for both cultivars at 1 DoT was followed by a decrease which at 7 DoT only in UB resulted in lower hexose levels for the chilled plants compared to the controls (Fig. 1 B). SW exhibited a similar reversal of hexose levels between chilled and control plants, which however was postponed to 21 DoT. The Suc levels were generally higher in UB at both temperatures compared with SW (Fig. 1 D). However, the cv effect was also dependent on temperature. Under chilling, Suc levels increased already on the first day of treatment, while the increase was much stronger in UB so that at 3 and 7 DoT the differences between cultivars were larger under chilling compared to control temperature. Starch levels increased in response to chilling transiently until 7 DoT with a much stronger response of UB (Fig. 1 F). A cultivar effect on starch was present on 3 and 7 DoT with higher levels in UB at both temperatures.

In addition to absolute concentrations, carbohydrate ratios between Suc and hexoses, and between source and sink organs, respectively, give a more precise view on the chilling impact on the dynamic equilibrium between the carbohydrate fractions and carbohydrate distributions between organs. The sucrose/hexose ratios showed cultivar-specific differences and responded to chilling with distinct reaction patterns between source and apex. Exposure to chilling caused increasing Suc/hexose ratios in the source leaves, with a stronger increase in SW (Fig. 2 A). In the apices of both cvs, the Suc/hexose ratio was increased by chilling until 7 DoT and then remained on similar levels. In contrast to the source leaves, however, ratios were higher in apices of UB than of SW and this effect was more pronounced on the condition of chilling (Fig. 2 C). The apex-source leaf ratios of carbohydrates were reduced in both cultivars under chilling. Under both temperature regimes, however, the apex-source leaf ratios of Suc were several fold higher in UB than in SW (Fig. 2 B). The hexose ratios between apex and source were in both cvs reduced on chilling conditions, but the reduction was smaller in UB (Fig. 2 D).

Photosynthesis and invertase activities as affected by cv and mild chilling

Since photosynthesis provides the bottleneck for the input of organic carbon into the plant and can be impaired at low temperatures, we analyzed the net photosynthetic rates of both cvs. Referring to the leaf dry weights of the measured plants, net photosynthetic rates were significantly lower in SW compared to UB at both temperatures (ca. 14 - 24 % at control temperature and ca. 13 - 32 % under exposure to chilling). No chilling response was found, however, on light conditions of 50 µmol m^{-2} s^{-1}, which complied with the light conditions during the growth of plants (Fig. 3).

Invertases play a crucial role in the regulation of carbohydrate metabolism particularly in relation to sink activity. They are also involved in plant reactions to several stress stimuli. Thus, we investigated the activities of vacuolar (vacINV), cytoplasmic (cytINV), and cell wall bound invertases (cwINV). The invertase activities, represented in Fig. 4, were measured on standard conditions to provide high turnover rates for the detection of acclimation of invertases to chilling stress [26]. The source and sink organs of both cvs displayed strong general cultivar-specific differences, which were more pronounced in the apex. However, only at a few dates a chilling response was detected (Significance Table in Fig. 4). UB showed generally higher activities of vacINV (Fig. 4 A) and cytINV (Fig. 4 C) in source leaves during the first week. Later on, the activity of both enzymes was slightly reduced at 21 DoT on chilling conditions in both cvs, without cultivar effects. Differences in cwINV activities were only found at 7 DoT (Fig. 4 E), when the activities were increased in response to chilling, with a more pronounced increase in UB.

Constitutivly higher invertase activities in UB were more obvious in the apex. The apical vacINV activities were higher in UB at both temperatures from 0 DoT until 3 DoT (Fig. 4 B). A chilling

induced reduction of activities was detected from 3 DoT until 21 DoT, which was stronger in UB at 21 DoT. The activities of cytINV showed strong general cultivar-specific differences throughout the experiment, with higher activities in UB compared to SW at both temperatures (Fig. 4 D). A chilling response was only found on 21 DoT, indicated by reduced activities. Apical cwINV activities also showed strong cultivar-specific differences, with general higher activities in UB (Fig. 4 F). Nevertheless, no significant response to chilling was found.

Phytohormone levels as affected by cv and mild chilling

Since it is known that the homeostasis of the phytohormones jasmonic acid (JA), abscisic acid (ABA), and of the auxin indole-3-acetic acid (IAA) plays an important role in cold stress responses, concentrations of these phytohormones were analyzed. The stem serves as a key transport unit for phytohormones, and hormones like IAA may control stem elongation. In addition to the source and sink organs, the uppermost part (1.5 cm) of the stem, directly located below the apex, was therefore, analyzed. No effect of chilling on JA levels could be detected in the source leaves and in the apex, because most JA values were below limits of quantification of 1.5 pmol per injection [34] for both temperature treatments (data not shown). JA levels in the stem were still low but detectable with slightly higher levels for SW at 1, 3 and 21 DoT. Chilling reduced the JA levels in both cvs only at 21 DoT (Fig. 5). We found that ABA levels in the source leaves, the upper stem and especially in the apex showed general cv specific differences, but ABA was not affected by the factor “temperature” or its interaction with the factor “cultivar” (Significance Table in Fig. 6). In the apex, ABA levels in UB were continuously more than twice as high as in SW at both temperatures (Fig. 6 A). Higher ABA levels for UB were also found in the upper stem at 21 DoT (Fig. 6 B) and in the source leaves at 1, 7 and 21 DoT with strongest differences at 21 DoT (Fig. 6 C). The IAA levels showed cultivar differences only in the first days, and a chilling reaction was detected only in the stem (Significance Table in Fig. 6). Independent of temperature, UB revealed higher IAA levels in the apex at 1 and 3 DoT (Fig. 6 B), in the upper stem at 0, 1 and 3 DoT (Fig. 6 D), and in the source leaves at 1 DoT (Fig. 6 F). IAA levels in the upper stem of both cvs were reduced after 21 days of chilling independent of the cultivar, (Fig. 6 D).

Gene expression as affected by mild chilling and cv

Samples were taken at three dates (3, 7, 21 DoT) to analyze differences in gene expression under the influence of the two temperature treatments. Additional files 3 and 4: Table S1A and Table S1B provide a complete overview of all annotated genes on the microarray. In a first step, we analyzed the number of genes, which were differentially expressed under chilling compared to control conditions in dependence on the cultivar (Fig. 7). Generally, overlapping of genes regulated in both cvs in the same direction was very low particularly in the apex. Also the total number of chilling-regulated genes was lower in the apex compared to the other organs particularly in SW. Both cvs showed a fast

reaction of gene expression to chilling in the apex with a prominent down-regulation at 3 DoT followed by a prominent up-regulation of genes at 7 DoT, while especially at 3 DoT more genes were affected in UB (Fig. 7 A). In the stem and the source leaves (Fig. 7 B, C), however, the up-regulation of genes in both cvs indicates three phases; a strong fast reaction at 3 DoT, a decrease of regulated genes after one week, and a higher number of up-regulated genes again at 21 DoT. Compared to the apex, the stem of both cvs showed a general higher number of differentially regulated genes with more down-regulated genes between 3 and 7 DoT, but less at 21 DoT in UB when compared with SW (Fig. 7 B). The highest numbers of up- and down-regulated genes in the source organ was observed for UB at 3 DoT and at 21 DoT and for SW at 7 DoT and 21 DoT (Fig. 7 C).

In the next step, we directly compared the hybridization signals of genes between the two cvs at control or chilling temperature, respectively. The analyzed genes were grouped into two different categories: 1) genes with chilling-independent cultivar-specific hybridization signals (C category), and 2) genes with chilling-dependent cultivar-specific hybridization signals, which showed cultivar dependent hybridization differences only under chilling exposure (CC category). A high number of genes showed general C category differences between both cultivars at both temperatures in all organs at all dates (compare column D in Additional File 5: Table S1C). These differences are probably based on sequence polymorphisms between the two cvs, while differences that are not constitutively present at all dates and in all organs are probably based on differential RNA accumulation.

Figs. 8 - 10 illustrate the numbers and functional groups of genes (CC category), which showed a cultivar-specific array hybridization only under the condition of chilling, indicating differential expression. In the apex (Fig. 8) and the stem (Fig. 9), the numbers reflect the strongest differences in gene expression during the early phase of the chilling response. In the apex, this phase is followed by fewer cultivar-specific differences at 7 and 21 DoT, whereas in the stem a similar response at 7 DoT is followed by a subsequent rise in number of cultivar-specific chilling responding genes at 21 DoT. However, prominent functional groups of genes with chilling-dependent expression patterns during the early phase mostly showed also responses during the later phases even though at lower levels. Such groups included genes related to “biotic stimuli”, “auxin metabolism and perception”, “antioxidative metabolism and perception”, “cell wall”, “gene expression and RNA metabolism”, “membrane transport”, “protein synthesis and degradation”, “secondary metabolism”, “signaling” and “miscellaneous”. In most cases, the different chilling responses between the cultivars involved up- and down-regulation of genes. Particular chilling-dependent genes related to “auxin metabolism and perception”, however, showed higher RNA accumulation levels in UB at 3 and 21 DoT in the apex (Fig. 8) and at 7 and 21 DoT in the stem if compared with SW. By contrast, chilling-dependent genes related to jasmonate mostly showed lower expression in UB. This also applies to genes of the group “biotic stimuli” at 7 and 21 DoT in the apex and 3 and 7 DoT in the stem, as well as to the categories

“membrane transport” and “mineral nutrient responsive and acquisition” at 3 DoT in the stem. In the source organ (Fig. 10), similar numbers of chilling-dependent cultivar-specific genes were identified at the three phases of chilling and the same functional groups were involved as found for apex and stem. In most cases, similar numbers of genes, up- and down-regulated by chilling, were found comparing UB and SW. At 3 DoT, however, functional groups such as “gene expression and RNA metabolism” or “signaling” showed more chilling-induced genes in UB than in SW, whereas the opposite picture was observed at 21 DoT for functional groups such as “Biotic stimuli”, “Cell wall”, “Membrane Transport” and “Signaling”. In source leaves, cultivar-specific genes related to ABA and jasmonate showed mostly lower RNA accumulation levels on chilling conditions in UB compared with SW.

Additional files 3 and 4: Table S1A and Table S1B show all expression values of genes hybridized with probes derived from UB and SW, respectively. Additional file 5: Table S1C shows the M-values of the UB/SW hybridization signal ratios for each temperature and date for those genes which exhibited significant differences. Here we focus on individual genes, which can be associated with the above-described metabolic responses and/or have putative functions in stress response. Fig. 11 shows the UB/SW M-values of these selected genes for the different organs, dates and temperature conditions.

Only a few photosynthesis-related genes were differentially hybridized between both cvs. Genes putatively involved in photosynthetic electron-transport coding for light harvesting complex gene and for plastocyanin-like domain-containing protein [40] showed, at least with one copy, continuously lower hybridization signals in all organs of UB. A gene coding for the chloroplast PIFI (postillumination chlorophyll fluorescence increase) protein, involved in the chlororespiratory electron transport [41], seemed to be chilling-induced in the cv UB. Among the families of genes coding for the small subunit of the carbon fixation protein RuBisCo (small subunit) and for its activase one member encoding the small subunit showed a lower expression in the stem of UB at 3 and 21 DoT. Genes for sugar transporters were mostly not differentially expressed, but two genes coding for glucose-6-phosphate/phosphate translocators and one for a carbohydrate transmembrane transporter/sugar:hydrogen ion symporter showed higher expression in the source organ of UB at least at two from three dates under both chilling and control conditions. In addition, several genes coding for invertase/pectin methylesterase inhibitor family protein showed lower expression in the apex, source and stem organ of UB when compared with SW under both temperature conditions, while a cell wall invertase gene seemed to be constituently higher expressed in the stem organ. Although sucrose and starch levels were different (Fig. 1) genes coding for starch synthase and sucrose (phosphate) synthase showed almost no different hybridization signals between both cvs.

With reference to the strong response of carbohydrate metabolism, several other genes coding for enzymes regulating the glycolysis were differentially regulated. A gene coding for hexokinase, catalyzing the initial step of glycolysis, was continuously lower expressed on chilling in the source leaves and the stem, but not in the apex of UB. On the same condition, a gene coding for fructose-bisphosphate aldolase showed higher expression levels in UB at 7 DoT in the apex and the source organ and at 3 and 7 DoT in the stem. Further, one gene coding for triosephosphate isomerase was higher hybridized in all three organs indicating gene polymorphism, while three others were higher expressed in the stem of UB. One gene coding for phosphoglycerate/bisphosphoglycerate mutase family protein showed higher expression in the apex of UB after 3 days of chilling and in the stem at all dates. In contrast, some genes encoding enzymes of the final steps of glycolysis showed lower hybridization levels in UB: Two genes coding for 2-phosphoglycerate dehydratase, an enolase catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP), showed this effect on chilling in all three organs of UB, but two genes coding for pyruvate kinase, which regulates the final irreversible and activity-regulating step of the glycolysis, at both temperatures in the source leaves of UB at 7 and 21 DoT and on chilling in the stem at 3 DoT. Enzymes of the citric cycle and its connection to the glycolysis partly showed ambivalent hybridization rates in UB compared to SW. While genes coding for PEP carboxylase were hardly differentially regulated on chilling, a PEP carboxylase kinase-encoding gene was up-regulated on chilling in UB in the apex and the stem and at 21 DoT in the source. Additionally, a gene coding for the antioxidant thioredoxin, which may have also regulatory functions in carbohydrate metabolism, showed several times more intense hybridization signals in UB compared to SW. One gene coding for the enzyme aconitase showed constitutively higher hybridization in UB. A gene coding for the enzyme fumarase showed higher expression in the stem of UB, and a malate dehydrogenase encoding gene was constitutively lower hybridized in all organs.

Furthermore, some genes coding for the metabolism and perception of stress related phytohormones showed distinct hybridization patterns. ABA-related genes displayed mainly general cultivar specific differences. Thus, one gene coding for carotenoid cleavage dioxygenase, involved in ABA biosynthesis, showed constitutively more intense hybridization signals in UB. Additionally, three genes coding for 9-cis-epoxy-carotenoid dioxygenase (1, 3) showed higher expression levels in the apex and the stem of UB at distinct dates after exposure to chilling. Two ABA-responsive genes coding for ABA-stress-ripening proteins showed divergent reactions: one gene showed constitutively stronger, the other one weaker hybridization signals in UB compared to SW. Several genes putatively coding for 1-aminocyclopropane-1-carboxylate oxidase controlling the final step of ethylene biosynthesis were lower expressed in UB at particular dates under both temperatures, while on chilling stress the most stable effect was found in source leaves and the stem. However, such consistent cultivar effect was not found for the family of genes encoding aminocyclopropane-1-

carboxylate synthase (Fig. 11). Differences in hybridization signals for genes encoding ethylene-responsive transcription factors were also ambivalent, some genes showed higher and others revealed lower levels in UB compared to SW (Additional file 5: Table S1C). Considering auxin related genes, one auxin efflux carrier gene was higher expressed in the source leaves of UB at both temperatures. One auxin:hydrogen symporter gene showed higher RNA accumulation levels in UB on chilling, and this was true in the source leaves at all dates and in the stem at two dates. Two genes coding for IAA amidohydrolase or IAA-amino acid hydrolase, the latter releases active IAA from conjugates, were higher expressed at 3 and 7 DoT in the apex of UB on chilling. Further, a gene coding for an Aux/IAA protein, a member of a family of transcriptional repressors, was constitutively down-regulated in UB on chilling. Several genes encoding auxin-induced SAUR (*SMALL AUXIN UP RNAs*)-like proteins showed higher expression levels for UB compared to SW under both temperatures, particularly in the apex and the stem. Several other auxin responsive proteins or transcription factors were also differentially regulated. Some of them showed higher, but others lower expression on chilling in UB. However, more differences were found in the apex and the stem compared to the source leaves (Additional file 5: Table S1C). Genes coding for enzymes of the JA biosynthetic pathway as 12-oxophytodienoate reductase, beta-hydroxyacyl-ACP dehydratase, chloroplast allene oxide synthase and lipoxygenases showed mainly lower hybridization signals in UB at both temperatures. Interestingly, among the genes related to the gibberellin (GA) pathways, several genes coding for gibberellin 20 oxidases, a rate-limiting enzyme for biosynthesis of active GAs, were in UB higher expressed in the apex. In the upper stem, however, only one of these genes showed this pattern, while several other members of this family showed the reversal cultivar-dependent effect. Furthermore, two genes encoding a gibberellin 2-beta-dioxygenase and a gibberellin 2-oxidase, which both can expected to contribute to reduction of the pool of active GAs, were higher expressed in the apex and upper stem of UB (Fig. 11). Comparing UB with SW, one gene encoding the GA receptor GID1 showed generally higher expression in apex and stem, whereas one other GA receptor GID2-encoding gene showed generally lower hybridization signals.

Several genes coding for heat shock proteins (HSP) showed cultivar-specific hybridization signals. Likewise, a gene coding for a chloroplast small heat shock protein class I was in the apex and the source mostly lower expressed in UB than in SW on control and chilling conditions (Fig. 11). One gene of a mitochondrial small heat shock protein was constitutively higher transcribed in UB in the upper stem. Furthermore, two genes coding for one low temperature and salt responsive protein and one drought-responsive family protein showed generally lower hybridization signals in all organs of UB. One dehydrin gene was lower expressed in the apex, but higher in the stem at 3 and 7 DoT. Three other genes of cold-regulated proteins were lower expressed in UB at particular dates mostly on chilling.

In our previous study [26], we found several chilling-stress-related transcriptions factors (TFs) that were affected by the mild chilling that we applied. Half of these TFs did not show any differential hybridization patterns between UB and SW for the three dates in the three organs on chilling. The remaining were partly differentially expressed but some higher and some lower in UB on chilling and no clear common response could be detected (Additional file 5: Table S1C). Only two genes, which can be related to the CBF/DREB pathway were found to be differentially regulated between the two cvs. The transcription factor ERF029 (similar to DREB1D) was only in the source leaves at 7 DoT lower expressed in UB under chilling, while a gene coding for the CCAAT-binding transcription factor CBF-B/NF-YA family protein showed higher RNA accumulation levels in UB in the source organs at all dates and at 21 DoT in the stem on chilling.

Effect of applications of ABA and nordihydroguaiaretic acid on growth and ABA levels under control and chilling conditions

Considering the constitutively higher ABA levels in the growing apical meristems of the tolerant cv UB at both temperatures, which were further associated with a higher hybridization of one carotenoid cleavage dioxygenase-encoding gene, we tested the growth response of both cvs to a pharmacological modification of endogenous ABA levels. In the first approach, we applied ABA in three concentrations to the chilling-sensitive cv SW. In other studies, spray applications of concentrations between 200 and 2,000 mg of physiologically active S-ABA per L to petunia enhanced shelf life and reduced time to wilting under water deficit stress, however, from 500 mg/L onwards ABA also reduced the number of flowers in non-stressed plants [42–44]. We therefore applied S-ABA at concentrations of 30, 120 and 480 mg/L (0.11, 0.45 and 1.82 mM). In the alternative approach, we aimed to inhibit the ABA synthesis in UB by exogenous application of an ABA synthesis inhibitor, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) [45]. Considering findings of Han et al. [46] and Ren et al. [47], we used concentrations of 15.1, 30.2 and 60.5 mg/L (0.05, 0.10, 0.20 mM). We analyzed the growth under control and chilling conditions and measured the ABA levels in the shoot apices at the end of the experiment.

Applications of ABA to SW decreased fresh and dry weight production of SW on control and chilling conditions, and the effect became stronger with increasing concentrations (Fig. 12 A, C). The ABA effect was, however, dependent on the temperature: The application of the lowest tested ABA concentration (0.11 mM) to SW reduced dry weight production only at 16 °C but not at 12 °C (Fig. 12 C). This significantly reduced the chilling-induced growth depression in absolute (Fig. 12 E) and relative terms (Fig. 12 G). A similar effect was observed for fresh biomass, even though not statistically significant. Analyzing different structural parameters revealed that ABA application enhanced the number of leaves only on chilling conditions and the relative leaf development rate on chilling compared to control conditions (Additional file 6: Figure S3 A, C, E; Additional file 7: Figure

S4 A). Fresh weight production of UB at control temperature was increased by the highest NDGA dosage of 200 mg/L (Fig. 12 B). However, dry weight production was not significantly influenced by NDGA-treatment even though it was slightly enhanced by NDGA on control conditions (Fig. 12 D). Nevertheless, chilling-induced depression of fresh and dry weight production of UB in absolute and relative terms was increased by NDGA already at the application of 50 mM (Fig. 12 F, H). However, the investigated structure parameters did not show any response to the NDGA-treatment (Additional file 6: Figure S3 B, D, F; Additional file 7: Figure S4 B). The analyses of ABA levels confirmed the constitutively higher ABA level in apices of UB compared with SW (Fig. 13). The apices of the ABA-treated SW plants showed a dose-dependent high accumulation of ABA (Fig. 13 A), especially at 12 °C, while even the lowest dosage enhanced the ABA concentration far above the constitutive level in UB (Fig. 13 B). The apex samples for the phytohormone measurements were taken one week after the last spray-treatment expecting that the newly grown tissue was not directly exposed to spray application. Considering the reduced leaf development rate at the reduced temperature (Additional file 7: Figure S4 A), the extremely high ABA levels in the chilled plants, which were reached after high ABA dosages, may be, however, partially based on some remains of the applied ABA on the surface of sampled apices. Nevertheless, the NDGA treatment reduced the ABA levels in apices of chilled UB plants to such an extent, that at the highest NDGA dosage ABA levels in UB were found to be similar to those measured in non-chemically treated plants of SW.

Discussion

Smaller growth depression of UB under mild chilling stress is related to constitutive and chilling-induced differences in gene expression

In previous studies we have shown that the chilling response of the sensitive *P. hybrida* cv SW consists of a complex derangement of the plants' functional integrity at the levels of carbohydrate metabolism, phytohormone homeostasis, and gene expression [26]. In the present comparative study, the growth of the cv UB, measured by dry and fresh weight production, was similar under control temperature but significant less disturbed by exposure to chilling compared to SW. The results of the detailed analyses of the two cvs at metabolic, phytohormone and transcriptome level at the two temperatures suggest that the higher chilling tolerance of the cv UB is based on a more balanced functional homeostasis. This provides a higher stability of utilization sinks compared to SW, which is largely maintained during the exposure to sub-optimal temperature.

Only few genes were simultaneously up- or down- regulated on chilling in both cvs (Fig. 7). Most genes affected by chilling were differentially regulated only in one of both cvs. In contrast, a comparative study of two distinct chilling-tolerant rice cvs found high numbers of common differentially regulated genes under exposure to low temperatures. Here, at least half of the genes

were affected in both cvs [48]. The more consistent chilling reaction might be due to the lower temperature of 4 °C, the authors used as cold treatment in the rice experiment, causing a more dominant temperature effect. Nevertheless, our data show that gene expression was differentially regulated between cvs, especially in the early phase of chilling in the source organ, which is responsible for the maintenance of carbohydrate production and thus for the energy supply.

In a second approach, we directly compared the hybridization rates of both cvs for each temperature. In this context, differences in signal intensities on the arrays between the two cvs might be based on different transcription levels but also on differential hybridization resulting from sequence polymorphisms of encoding genes. A high number of annotated genes were found with constitutive differential hybridization rates in all organs at both temperatures and at all dates (C category, compare column D in Additional file 5: Table S1C). These constitutive differences might probably be the result of gene polymorphisms between the both cvs, while differences in hybridization rates that were not observed continuously in all organs at both temperatures and at all dates might indicate differences in gene expression.

General differences in gene expression and metabolism might contribute to the potential of a plant to cope with stress conditions. Nevertheless, distinct expression patterns provoked after exposure to chilling should be also considered to estimate the cultivar-specific plasticity in response to the sub-optimal temperature. The expressional response of genes, which were only differentially chilling-regulated between both cvs, featured organ specific patterns. After differential apical chilling responses in the early chilling phase, the individual differences attenuated (Fig. 8), indicating a harmonization of apical gene regulation between both cvs with increasing duration of chilling. In contrast, the differential chilling responses of the stem and source organs between both cvs were divided in a fast and a long-term response. While different responses in the stem were only transiently attenuated after one week (Fig. 9), in the source organ the quality of involved functional groups merely changed (Fig. 10). It seems that the source organ of UB responds faster to chilling indicated by more chilling-induced genes (Fig. 7) and higher hybridization signals (Fig. 10) during the early phase.

Higher tolerance of UB to mild chilling is associated with a more stable leaf carbohydrate metabolism and a generally higher abundance and utilization of sucrose in the shoot apex

The carbohydrate metabolism is the indispensable basis for plant growth, reliant on the availability of carbohydrates at the utilization sinks through the source-sink-transport from carbon sources. A previous study has proven that the 4 K temperature reduction we applied is sufficient to disturb the carbohydrate homeostasis and to cause substantial changes in the source and the utilization sink organs of the chilling-sensitive cv SW [26]. In order to test the hypothesis that the regulation of the carbohydrate metabolism might differ between distinct chilling-tolerant cvs, we monitored

carbohydrate levels at both ends of the source-sink network, at the uppermost fully developed leaves as carbohydrate source organ, and at the apex as utilization sink.

Under control conditions, source leaves of both cvs contained similar sugar levels. However, under chilling the cv SW accumulated much higher sugar levels than the cv UB (Fig. 1 A, C), while the sucrose fraction increased (Fig. 2A). In general, an increase of levels of soluble sugars can be interpreted as a beginning chilling protection response [4]. Thus, Stitt and Hurry [49] showed that a consecutively increased expression and post-translational activation of enzymes of the Suc synthesis pathway seemed to counteract cold-induced inhibition of Suc synthase and supported the plants' acclimation to low temperature as well as a modified expression of Calvin cycle enzymes. In addition, Usadel et al. [4] observed a repression of genes for Suc and starch breakdown with declining temperatures (4 °C) in *Arabidopsis* leaves. The increased sucrose levels in response to chilling in our study seemed not to be the result of altered expressions of sucrose phosphate synthase or sucrose synthase. Thus, the different levels may be influenced by post-transcriptional regulation of Suc synthesis and degradation. Nevertheless, it has also to be considered that genes which are not represented on the array might play a role.

The higher sugar levels in leaves of SW at chilling cannot be explained by a higher net photosynthetic rate. By contrast, the general lower net photosynthetic rates in SW on a leaf dry weight basis at both temperatures indicate a general lower potential of the sensitive cv for carbon assimilation (Fig. 3). The gene expression analysis did not elucidate putative genes that might be responsible for these differences in photosynthetic rates. Many other reasons including post-transcriptional regulation of essential proteins or stomata regulation and density may be involved. The decrease in temperature from 16 °C to 12 °C did not affect the net photosynthetic rate of both cvs. It has to be taken into account, that light and dark respiration show higher temperature optima than gross photosynthesis. That means that at higher temperatures a larger amount of the photosynthetic CO^2 fixation is compensated by respiratory CO^2 loss than at lower temperatures. [50]. The present results stay in accordance with findings of Klopotek et al. [51]. In their study with the *P. hybrida* cv 'Mitchell', the net photosynthesis of cuttings at a PPFD of 100 µmol $m^{-2}s^{-1}$ was almost not affected by temperature when increased from 14 °C up to 30 °C, whereas dark respiration showed a strong increase. However, the higher expression of three genes in source leaves of UB encoding one carbohydrate transmembrane transporter/sugar-hydrogen ion symporter and two glucose-6-phosphate/phosphate translocators (Fig. 11) might indicate a better intracellular and long-distance sugar distribution for UB compared to SW. Athanasiou et al. [52] supposed that the glucose-6-phosphate/phosphate translocator plays a signaling role by influencing the partitioning of sugar phosphates between the chloroplast stroma and the cytosol or altering the phosphate balance of the cell. Sugar-hydrogen symporters have two important functions, they play a crucial role in the stomata-opening function of guard cells that

antagonizes the ABA-induced closure of stomata [53] and they are involved in phloem loading [54]. Taken together, these expression patterns suggest a wider stomata opening despite the higher ABA levels in the source leaves and an improved consecutive translocation of sugars from the chloroplasts of source leaves to the cytosol and to the phloem in UB. A better carbon supply of source leaves by open stomata and increased removal of photosynthetic products might have contributed to the observed higher photosynthetic rates and obviously enhanced sucrose transport in UB at both temperatures.

An improved Suc-translocation is supported by the finding that UB accumulated less of the main carbohydrate transport form Suc in the source leaves under exposure to chilling, but more in the apex compared to SW (Fig. 1 C, D; Fig. 2 A, B, C). Since the photosynthetic rates were higher in UB, the lower sugar levels in source leaves under chilling seem to indicate that UB exports carbohydrates from source leaves to the shoot apex more efficiently than SW. Interestingly, UB revealed constitutively higher activities of cytosolic and cell wall invertases in the apex compared to SW (Fig. 4). Invertases play a general important role in the sink activity in petunia [33,55,56]. Cell wall bound invertases in particular are responsible for sucrose partioning by unloading of sucrose into cells [57]. Thus, the general cultivar-specific higher cell wall invertase activities suppose an enhanced influx of Suc and in consequence a constitutively higher carbohydrate supply of the UB apex. Higher sucrose levels in the apex, particularly at chilling, might have, however, indirectly caused the higher cytosolic and vacuolar invertase activities via feed-forward mechanisms. Thus, even independent of carbohydrate utilisation at the sink site, the sugar export from leaves and/or the transport route between source leaves and the apex may be more efficient in UB compared to SW. The higher concentrations of sucrose and the higher activities of invertases that hydrolyze Suc into Glc and Fru provide osmolytes that rise the osmotic pressure in the cells of the apical meristem. This might power an osmotic potential gradient as possible driving factor for cell elongation and plant growth [58]. In the apices of the two cvs, only small differences were found for transcripts of genes coding for invertases particularly at chilling (Fig. 11). In contrast, transcripts of several genes encoding invertase inhibitors showed lower expression in UB than in SW, further depending on the organ and days of temperature acclimation (Fig. 11). Invertase inhibitor proteins seem to be detached from cwInv during enzyme extraction, and high sucrose concentrations in the enzyme assays might protect invertases from re-inhibition [15]. We therefore suppose that the measured differences in invertase activities are more due to a generally higher quantity of invertases in UB apices than due to a distinct post-transcriptional regulation. Consequently, a reduced post-transcriptional regulation of invertase activities in UB compared to SW due to less inhibition would mean even higher *in vivo* differences of activities than the measured ones.

The microarray results further indicate that the carbohydrate metabolism of UB differs from that in SW by being more anabolic orientated. Genes related to glycolysis and citric cycle showed more general hybridization differences between both cultivars than distinct reactions to chilling. Of particular interest is the higher expression of a gene coding for the PEP carboxylase kinase in the apex and the stem and in the late phase in the source leaves of UB. This kinase activates the enzyme PEP carboxylase by phosphorylation [59]. The C4-form of PEP carboxylase kinase seems to be redox-regulated by thioredoxin [60]. If this should also apply to the C3-form of PEP carboxylase kinase, the constitutive higher hybridisation of a thioredoxin gene could indicate an altered regulation of PEP carboxylase via an additional altered activation of PEP carboxylase kinase through thioredoxin. In contrast, genes coding for PEP carboxylase itself were not higher expressed in UB. In C3-plants like petunia, a main function of PEP carboxylase is to increase the influx from glycolysis to the citric acid cycle by converting PEP to oxaloacetate [61,62]. The constitutivly higher hybridizations of a gene encoding for aconitase, an enzyme down-stream from oxaloacetate in the citrate cycle, indicate a polymorphism of this gene between both cvs. Should this contribute to a more active enzyme, it would also enhance the citrate cycle flux in UB. Further, the constitutive lower hybridization of a malate dehydrogenase-encoding gene seems also to indicate a polymorphism. The resulting enzyme replenishes the oxaloacetate pool. A lower expression of this gene and/or lower activity of the encoded protein would suggest a higher need for carbon influx at the oxaloacetate step. On the other hand, an increased flux in the cycle in combination with a restriction of the oxaloacetate replenishment might indicate higher carbon fluxes to cataplerotic pathways and by that towards biosynthetic processes determining growth. The lower expression for a hexokinase gene, which catalyzes the initial step of glycolysis, especially the general ones in the source leaves and the stem, might indicate a reduced glycolysis in both organs. In the source organ, this may have contributed to a higher abundance of sugars for carbohydrate export. However, while in the apex hexokinase was not affected at chilling, 2-phosphoglycerate dehydratase, an enolase, catalyzing the conversion of 2-phosphoglycerate to PEP, was in UB lower expressed in all organs under chilling compared with SW. A reduced PEP synthesis may provide a bottleneck of carbon input for the citric cycle. We have, however, to consider that the constitutively higher invertase activities in UB, together with the high abundance of sucrose, might provide a high concentration of substrate for glycolysis so that PEP synthesis may be still higher compared to SW. Alternativly, the above-described enhancement of citric cycle might also be a way to channel more of a scarce resource, PEP, to the citric cycle for more biosynthesis. In UB source leaves, also a gene coding for pyruvate kinase was lower expressed at 7 and 21 DoT. While pyruvate kinase catalyzes one of the energy-generating reactions of the glycolysis, a putatively reduced activity of this enzyme in source leaves of UB might give a hint at a lower energy consuming activity of UB compared to SW.

Taken together, the sensitive cv accumulates at chilling the sugars preferentially in the source leaf, whereas the sucrose abundance and utilization in the shoot apex is low probably based on low export of sucrose out of the source. By contrast, the more tolerant cultivar reveals a less disturbed leaf sugar homeostasis and a generally higher abundance and utilization of sucrose at the sink site. Recently, we [26] considered the carbohydrate metabolism response of SW as part of a primary defense strategy to protect the source leaves, which have an important maintenance function for the plant. By contrast, the less disturbed carbohydrate metabolism in the source leaf but higher and more stable carbohydrate abundance and utilization in the sink of UB indicate that the chilling-tolerant cv follows a "growth priority" under the condition of mild chilling.

Auxin and constitutive higher ABA concentrations in the apex might contribute to chilling tolerance

Beside reactions to biotic stimuli like pathogen infection, JA is also known to be involved in reactions to drought, heat and cold stress [20]. Thus, jasmonate seems to enhance the freezing tolerance of *Arabidopsis.* While it controls the INDUCER OF CBF EXPRESSION (ICE)–C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 (CBF/DREB1) cascade, jasmonate works as important upstream signal in the ICE-CBF/DREB1 pathway in *Arabidopsis* that positively regulates freezing tolerance [21]. The tolerant cv UB revealed mostly lower JA levels in the upper stem at both temperatures (Fig. 5), which was further associated with lower hybridization signals for of JA biosynthesis related genes. This indicates that JA signaling, contrasting to its role in freezing tolerance, did not contribute to the higher tolerance to mild chilling stress of UB. In accordance to this view, we did not find any indication for an expressional chilling response of the jasmonate-dependent ICE-CBF/DREB1 cascade in none of the two cvs. Both findings combined indicate, that the ICE-CBF/DREB1 cascade was probably not yet fully activated at this mild chilling, neither in the tolerant nor in the sensitive cv.

Shibasaki et al. [63] supposed that cold-induced changes in plant development and growth might be related to intracellular auxin gradients, based on the inhibition of intracellular trafficking of auxin efflux carriers. The IAA levels in the apex and the upper stem were higher under both temperatures in UB during the first days after DoT (Fig. 6). The higher expression of a gene encoding an efflux carrier in the source leaves of the same cultivar might indicate that an enhanced auxin transport from leaves supported the higher IAA levels in the organs. IAA amidohydrolases and IAA-amino acid hydrolases release active IAA by hydrolyzing auxin storage conjugates [64]. Considering the higher expression of genes coding for both enzymes in the apex of UB, such processes might also have contributed to the higher IAA levels in UB during the first days. Even though the higher IAA levels in UB were not maintained after 3 DoT, they may have contributed to the higher tolerance to chilling. This view is supported by our finding, that genes coding for auxin-responsive SAUR proteins showed higher

expressions in the apex and the upper stem of UB under both temperatures. Considering recent publications providing evidence that SAUR proteins promote cell expansion involving activation of plasma membrane H^+-ATPases, Ren and Gray [65] proposed that SAURs are key effector outputs of hormonal and environmental signals that regulate plant growth and development.

One growth characteristic of UB in contrast to SW is the higher elongation of the main shoot at chilling (Additional file 2: Figure S2 A). With other plant species, active GAs and GA metabolism have been shown to control shoot elongation at low temperatures [66-69]. The differential expression of genes related to the GA pathways in UB compared to SW, which partially differed between the organs, may indicate that the GA homeostasis and signaling is also involved in the higher chilling tolerance of UB. The expression data particular of genes encoding gibberellin 20 oxidases suggest higher levels of active GAs in the shoot apex of UB. The entire expression pattern of all genes, however, indicates that the contrast of GA homeostasis between both cvs is rather complex. The finding that UB revealed higher expression of one gene encoding the GA receptor GID1 in apex and stem, suggests that UB exhibits higher GA signaling capacity in these tissues. According to a current model, GID1 functions as soluble GA receptor interacting with DELLA proteins as active repressors of GA response. GID1 initiates proteosomal degradation of DELLAs via enhancing recognition by the F-box protein of an ubiquitin E3 ligase SCF complex, thereby releasing the GA repression [70]. The protein GID2 is a component of the E3 ligase SCF complex. The additional finding, that one gene encoding a GID2 receptor was lower hybridized in UB than in SW may indicate the presence of a polymorphism between both cvs. That might be an indication that DELLA-dependent GA signaling pathways could be differentially used in the two cvs [71].

ABA biosynthesis in the root, but especially in the shoot enhanced chilling tolerance in tomato and was suggested to indirectly control endogenous ethylene levels that might raise tomato susceptibility to chilling stress [16]. Yang et al. [72] related an enhanced expression of ABA, auxin, and JA-related genes under cold exposure in the shoots and roots of rice to cold tolerance, scaled by leaf fluorescence and membrane leakage at 4 °C. In contrast, Mega et al. [73] found that already sustained low ABA levels in rice seedlings during cold (4 °C) could increase the seedlings vigor, while an excessive lack of endogenous ABA reduced cold tolerance. Thus, the general ABA levels, but also the homeostasis in the plants may play a role for chilling tolerance. In this context, the constitutive higher ABA levels particularly in apices of UB (Fig. 6) might have contributed to the higher chilling tolerance as reflected by the enhanced dry matter production under chilling and the enhanced relative growth at 12 °C versus 16 °C compared to SW. The data support the assumption that the higher ABA levels contributed to an effective stress response machinery particularly at the growing points of the plant, which are most important for the development of new plant material. The higher ABA levels in UB were associated with continuously higher hybridization rates of a carotenoid cleavage dioxygenase

gene in all organs. This enzyme is similar to the 9-cis-epoxycarotenoid dioxygenase, which catalyzes xanthophyll cleavage, the first and rate-limiting step of ABA biosynthesis [74]. The generally higher hybridization rate in UB might be due to polymorphism of this gene between the two cvs. We propose that the specific morphism of the carotenoid cleavage dioxygenase gene in UB provides an enzyme with higher biological activity contributing to the higher ABA levels in UB when compared with SW (Fig. 11). Levels of abscisic stress ripening proteins (ASR) can be increased by ABA and abiotic stressors. Thus, several *ASR* genes in tomato are induced by drought and cold [75]. ASRs play different roles. A chaperone-like activity was found for ASR1 in rice [76]. In contrast, some nuclear ASR proteins seem to bind to specific promoter sequences and modulate gene expression on transcriptional level [77]. While one ASR gene was constitutively higher and another lower hybridized in UB indicating polymorphism of the encoded regulatory proteins, it is unclear if this is involved in the chilling response. Nevertheless, the constitutivly higher ABA levels in UB and the complex differences in transcripts related to ABA biosynthesis and signaling suggest that differences in the ABA machinery contribute to the higher chilling tolerance of UB. Interestingly, ABA signaling has also a great influence on carbohydrate metabolism including regulation of invertases. Thus, ABA seems to have a key role in sucrose metabolism and starch biosynthesis by inducing genes for sucrose synthesis and degradation [78]. In contrast, ABA inhibits genes related to starch biosynthesis [79]. VacInv expression and activity was enhanced in maize seedlings by ABA-treatment [80,81] and ABA and sucrose increased the transcript levels of a cwINV [78]. Considering these data, both the constitutive higher cwINV activities and Suc levels in the apices of UB can be correlated with the constitutive higher ABA levels in the UB apex.

In non-stressed plants, normal ABA levels are essential for maintaining shoot development and leaf expansion. This maintenance function is partly reducible to a restriction of ethylene biosynthesis and/or sensitivity [82]. In addition, a low-water stress-induced accumulation of ABA seems to support root growth by restricting ethylene biosynthesis and/or sensitivity [83]. The role of ethylene in cold stress is more complex. Ethylene production declines quickly in reaction to cold. Since ethylene signaling appears to regulate negatively the CBF freezing tolerance pathway, a decrease in ethylene biosynthesis under chilling seems to be important for an activation of this pathway [84]. Thus, the lower expression rates of genes related to ethylene biosynthesis in UB compared to SW might be a consequence of the increased ABA levels and contribute to an enhanced chilling tolerance.

Manipulations of hormone levels in plants by pharmacological approaches may unravel hormone functions in regulation of growth and stress tolerance. However, it is generally difficult to obtain such an internal distribution of the hormone within the plant, which mimics the hormone homeostasis controlled by the plant itself in dependence on its genetic and response to the environment. This particularly applies to ABA, which on the one hand can increase stress tolerance depending on its

concentration but on the other hand may retard growth [85]. Exogenous application of ABA protected tomato plants from damage caused by suboptimal temperatures (day/night: 16/8 °C compared to 28/22 °C) [86]. This increased tolerance to chilling was defined by a reduced decline in chlorophyll content and in the net photosynthetic rate. ABA treatment also enhanced freezing viability of rice seedlings [87]. ABA can, however, reduce stomatal conductance and photosynthetic rate of leaves [88] and is also known to inhibit shoot growth and cell cycle progression [89]. Considering these relationships, we used a dual pharmacological approach to study the function of ABA in chilling tolerance of petunia. We applied ABA to the sensitive cv SW aiming to increase endogenous ABA and applied the inhibitor NDGA to the tolerant cv UB aiming to reduce the endogenous ABA level. Even though none of the applied concentrations of ABA did enhance absolute dry matter production of chilled plants of SW, the lowest application rate decreased the chilling-induced growth depression when compared to the growth at 16 °C (Fig. 12), which is one criterion for the higher chilling tolerance of UB compared to SW. Furthermore, the finding that ABA treatment at this level enhanced the number of produced leaves at chilling compared to non-treated plants indicates a growth-promoting influence of ABA at the sub-optimal temperature. This positive impact of ABA application on parameters of chilling tolerance in SW matched with the finding that applications of the ABA biosynthesis inhibitor NDGA to the tolerant cv UB enhanced chilling-induced growth depression. The ABA levels in the apices of the plants indicate that even the lowest ABA application raised ABA levels in SW above the control levels in UB. In UB, however, the highest application of NDGA decreased ABA to a level similar to the control levels in SW. It appears that the higher ABA level in UB contributes to the higher chilling tolerance but simultaneously restricts growth under non-stress conditions. As a whole, these application experiments support a protective role of ABA in chilling tolerance in petunia. However, considering the distribution of ABA between the different plant organs in UB compared to SW, this function seems not simply related to the overall ABA level in the whole plant but particular dependent on the fine-tuning of ABA homeostasis between particular organs. This regulation should be further investigated by complete sequencing of the candidate genes for characterization of polymorphisms and by modified expression while organ-specific promotors should also be considered. Exemplary, a constitutive overexpression of 9-cis-epoxycarotenoid dioxygenase, crucial for ABA biosynthesis, drastically enhanced drought resistance [90]. However, whereas the authors overexpressed the gene in source leaves, plant growth and development were affected due to decreases in stomatal conductance and photosynthesis. By the use of apical-meristem-specific promoters, 9-cis-epoxycarotenoid dioxygenase could be overexpressed in the apex to up-regulate ABA levels there locally, but the growth-retarding effects of ABA on the source leaves could be avoided.

CBF-pathway and heat- and cold-induced proteins seem not to have a primary role in tolerance of petunia to mild chilling

Only one gene related to the CBF-pathway, coding for a CCAAT-binding transcription factor CBF-B/NF-YA family protein, was higher expressed in the source leaf of UB at both temperatures. One other CBF-related gene was lower expressed at chilling at 7 DoT in the source leaf of UB. The data do not support an important role of the CBF-pathway for the higher tolerance of UB to mild chilling stress. In addition, the chilling-responsive transcription factors we found in a previous study on SW [26] did not feature a clear cultivar-specific pattern. Accumulation of small molecular heat shock proteins are known to be correlated with chilling tolerance. However, this usually applies to stronger stress situations compared to the present study. Thus, chilling tolerance in tomato, measured by chilling injury symptoms, was enhanced by the overexpression of a chloroplast-localized small molecular heat-shock protein [91]. Interestingly, in the present study one gene encoding a chloroplast small heat shock protein class I was in the apex and source leaves mostly lower expressed in UB compared with SW. Accordingly, other genes coding for cold-, drought- and salt-responsive proteins were lower hybridized in the tolerant cv. These findings support the concept that at mild chilling stress the tolerant cv follows a "growth priority" rather than a defense strategy.

Conclusion

Mild chilling reduces the production of fresh and dry weights of both cvs with a smaller impact on the chilling-tolerant cv UB. On the metabolic level, the exposure to chilling causes a derangement of the homeostasis of soluble sugars in both cvs, whereas the chilling-tolerant cv UB shows a more stable leaf carbohydrate metabolism and a generally higher abundance and utilization of sucrose in the shoot apex. This is correlated with an apparently improved carbohydrate translocation from source leaves towards the apical organ and a generally higher photosynthetic rate, while the photosynthetic rate is not affected by chilling itself. Higher cwINV and cytINV activities in the apex combined with a putatively reduced inhibition of invertase activities by invertase inhibitors might contribute to the sucrose import into and utilization in the apex. The array data further indicate an improved carbon flux from glycolysis into citric cycle driven by higher PEP carboxylase kinase levels and an enhanced citric cycle flux. A higher tendency towards cataplerotic metabolic pathways in combination with a higher osmotic potential in the apical organ, caused by the higher Suc abundance that is further increased by the enhanced invertase activity, might enhance cell elongation and plant growth at the growing points of UB. The observed general differences in phytohormone levels and the related array results support this higher growth priority of UB. While JA seems not to play a role in chilling tolerance at this mild stage of chilling stress, we suggest a key role for ABA homeostasis and signaling. A specific morphism of carotenoid cleavage dioxygenase gene in UB might provide a higher activity of this enzyme contributing to the general higher ABA level in the shoot apex, while ABA signaling might be at least partly responsible for the increased Suc levels and higher cytINV and

vacINV activities and further contribute to down-regulation of ethylene synthesis pathway. The functional role of ABA in chilling stress tolerance is supported by reduction and enhancement of chilling induced growth depression in response to treatments with ABA and the ABA synthesis inhibitor NDGA, respectively. However, organ-specific ABA homeostasis seems to be essential for proper growth performance. The microarray data further indicate that at the level of mild chilling the CBF-pathway, heat-shock proteins and cold-induced proteins do not have a dominant role for chilling tolerance of petunia. Further characterization and functional studies of candidate genes controlling the discussed metabolic and plant hormone pathways by use of mutants, RNAi technology and overexpression with involvement of organ-specific promotors are necessary to elucidate these relationships.

Abbreviations

ABA, abscisic acid; ACP, acyl carrier protein; ASR, abscisic stress ripening proteins; Aux, auxin; CBF/DREB1, C-REPEAT BINDING FACTOR/DEHYDRATION-RESPONSIVE-ELEMENT-BINDING PROTEIN; C category, genes with chilling-independent cultivar-specific hybridization signals; CC category, genes with chilling-dependent cultivar-specific hybridization signals; cv(s), cultivar(s); cwINV, cell wall bound invertase; cytINV, cytoplasmic invertase; DELLA, DELLA family of proteins, named after the conserved Asp-Glu-Leu-Leu-Ala N-terminal motif; DNase, desoxyribonuclease; DoT, days after differentiation of temperature; DW, dry weight; ERF, ethylene responsive factor; Fru, fructose; FW, fresh weight; GA, gibberellin; GC-MS/MS, gas chromatography-tandem mass spectrometry; GID1, GIBBERELIN-INSENSITIVE DWARF 1; GID2, GIBBERELLIN-INSENSITIVE DWARF 2; Glc, glucose; H+-ATPases, proton adenylpyrophosphatase; HSP, heat shock protein; IAA, indole-3-acetic acid; ICE-CBF/DREB1, INDUCER OF CBF EXPRESSION–C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1; JA, jasmonic acid; NDGA, nordihydroguaiaretic acid; n.s., not significant; PEP, phosphoenolpyruvate; pfp, probability of false prediction; *P. hybrida*, *Petunia hybrida*; PIFI, postillumination chlorophyll fluorescence increase; PPFD, photosynthetically active photon flux density; RNA, ribonucleic acid; RNAi, RNA interference; RuBisCo, Ribulose-1,5-bisphosphate carboxylase/oxygenase; SAUR, *SMALL AUXIN UP RNAs*; SCF, SKP1P-CDC53P-F-box protein; Suc, sucrose; SW, SweetSunshine Williams; TF, transcriptions factor; UB, Ultra Blue; vacINV, vacuolar invertase;

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAB assisted the design of the research, performed the research and analyzed the data except on photosynthesis and wrote the manuscript. SAO analyzed photosynthesis and edited the manuscript. PF assisted the design of research, contributed to the analysis of microarray data and edited the manuscript. UD designed the research and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Baerbel Broszies, Sabine Czekalla, Kerstin Fischer and Klaus Fricke (all IGZ) for their exceptional technical support in cultivating the petunias, sampling, extracting the RNA for the microarray studies and performing the phytohormone analyses. Further, we thank Florian Steinbacher (Technical University of Munich, Freising, Germany) for the realization of the measurements of photosynthetic rates. Furthermore, we thank Kathrin Schulz for substrate analyses and Dr. Siegfried Zerche (both IGZ) for helpful advices regarding the fertilization of plants. We thank Beate ter Hell and Dr. Dirk Ludolph from the Lehr- und Versuchsanstalt für Gartenbau Ahlem for their support in developing the chilling test conditions and Selecta Klemm GmbH & Co. KG (Stuttgart) for recommending the cvs and providing rooted cuttings.

Funding

The work was funded by the BMBF (Federal Ministry of Education and Research of the Federal Republic of Germany, 0315542E) within the BMBF-AgroClustEr: 'WeGa - Horticulture Research Network - Product and Production Safety in Intensive Plant Production Systems' and further supported by ministries of the state of Brandenburg, the Free State of Thuringia and the Federal Republic of Germany.

REFERENCES

1. BMEL. Verbrauch von Blumen und Zierpflanzen. In: Der Gartenbau in Deutschland - Daten und Fakten. Bundesministerium für Ernährung und Landwirtschaft [Internet]. 2014. Available: http://berichte.bmelv-statistik.de/GBB-0000100-2012.pdf. Accessed 05 Mar 2016.
2. Lindlöf A, Chawade A, Sikora P, Olsson O. Comparative transcriptomics of Sijung and Jumli Marshi rice during early chilling stress imply multiple protective mechanisms. PLoS One. 2015; doi:10.1371/journal.pone.0125385
3. Yamori W, Noguchi K, Terashima I. Temperature acclimation of photosynthesis in spinach leaves: analyses of photosynthetic components and temperature dependencies of photosynthetic partial reactions. Plant, Cell Environ. 2005; doi:10.1111/j.1365-3040.2004.01299.x

4. Usadel B, Bläsing OE, Gibon Y, Poree F, Höhne M, Günter M, et al. Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. Plant, Cell Environ. 2008; doi:10.1111/j.1365-3040.2007.01763.x
5. Mahajan S, Tuteja N. Cold, salinity and drought stresses: An overview. Arch Biochem Biophys. 2005; doi:10.1016/j.abb.2005.10.018
6. Venema JH, Posthumus F, van Hasselt PR. Impact of suboptimal temperature on growth, photosynthesis, leaf pigments and carbohydrates of domestic and high-altitude wild *Lycopersicon* species. J Plant Physiol. 1999; doi:10.1016/S0176-1617(99)80087-X
7. Roitsch T. Source-sink regulation by sugar and stress. Curr Opin Plant Biol. 1999; doi:10.1016/S1369-5266(99)80036-3
8. Allen DJ, Ort DR. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci. 2001; doi:10.1016/S1360-1385(00)01808-2
9. Druege U, Kadner R. 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 Biol Technol. 2008; doi:10.1016/j.postharvbio.2007.06.008
10. Yamori W, Noguchi K, Hikosaka K, Terashima I. Phenotypic plasticity in photosynthetic temperature acclimation among crop species with different cold tolerances. Plant Physiol. 2010; doi:10.1104/pp.109.145862
11. Yamori W, Noguchi K, Hikosaka K, Terashima I. Cold-tolerant crop species have greater temperature homeostasis of leaf respiration and photosynthesis than cold-sensitive species. Plant Cell Physiol. 2008; doi:10.1093/pcp/pcn189
12. Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK. Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot. 2003; doi:10.1093/jxb/erg050
13. Brummell DA, Chen RKY, Harris JC, Zhang H, Hamiaux C, Kralicek A V., et al. Induction of vacuolar invertase inhibitor mRNA in potato tubers contributes to cold-induced sweetening resistance and includes spliced hybrid mRNA variants. J Exp Bot. 2011; doi:10.1093/jxb/err043
14. Rausch T, Greiner S. Plant protein inhibitors of invertases. Biochim Biophys Acta - Proteins Proteomics. 2004; doi:10.1016/j.bbapap.2003.09.017
15. Bonfig KB, Gabler A, Simon UK, Luschin-Ebengreuth N, Hatz M, Berger S, et al. Post-translational derepression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. Mol Plant. 2010; doi:10.1093/mp/ssq053
16. Ntatsi G, Savvas D, Druege U, Schwarz D. Contribution of phytohormones in alleviating the impact of sub-optimal temperature stress on grafted tomato. Sci Hortic (Amsterdam). 2013;

doi:10.1016/j.scienta.2012.09.002

17. Anderson MD, Prasad TK, Martin BA, Stewart CR. Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance. Plant Physiol. 1994;105:331–9.
18. Yao GH, Gao PP, Wang YP, Wang LH, Xu GD, Liu P. Abscisic acid improves chilling-induced oxidative stress in *Trichosanthes kirilowii* maxim seedlings. J Agric Sci Technol. 2013;15:583–592.
19. Rahman A. Auxin: a regulator of cold stress response. Physiol Plant. 2013; doi:10.1111/j.1399-3054.2012.01617.x
20. Du H, Liu H, Xiong L. Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Front Plant Sci. 2013; doi:10.3389/fpls.2013.00397
21. Hu Y, Jiang L, Wang F, Yu D. Jasmonate regulates the INDUCER OF CBF EXPRESSION-C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 cascade and freezing tolerance in *Arabidopsis*. Plant Cell. 2013; doi:10.1105/tpc.113.112631
22. Akhtar M, Jaiswal A, Taj G, Jaiswal JP, Qureshi MI, Singh NK. DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. J Genet. 2012; doi:10.1007/s12041-012-0201-3
23. Chinnusamy V, Ohta M, Kanrar S, Lee B-H, Hong X, Agarwal M, et al. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev. 2003; doi:10.1101/gad.1077503
24. Thomashow MF. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol. 1999; doi:10.1146/annurev.arplant.50.1.571
25. Walworth AE, Song G, Warner RM. Ectopic AtCBF3 expression improves freezing tolerance and promotes compact growth habit in petunia. Mol Breed. 2014; doi:10.1007/s11032-013-9989-7
26. Bauerfeind MA, Winkelmann T, Franken P, Druege U. Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress. Front Plant Sci. 2015; doi:10.3389/fpls.2015.00583
27. Li B, Ning L, Zhang J, Bao M, Zhang W. Transcriptional profiling of petunia seedlings reveals candidate regulators of the cold stress response. Front Plant Sci. 2015; doi:10.3389/fpls.2015.00118
28. Druege U, Franken P, Lischewski S, Ahkami AH, Zerche S, Hause B, et al. Transcriptomic analysis reveals ethylene as stimulator and auxin as regulator of adventitious root formation in petunia cuttings. Front Plant Sci. 2014; doi:10.3389/fpls.2014.00494
29. Breuillin F, Schramm J, Hajirezaei M, Ahkami A, Favre P, Druege U, et al. Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. Plant J. 2010; doi:10.1111/j.1365-

313X.2010.04385.x

30. Hajirezaei MR, Takahata Y, Trethewey RN, Willmitzer L, Sonnewald U. Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. J Exp Bot. 2000; doi:10.1093/jexbot/51.suppl_1.439
31. Klopotek Y, Haensch KT, Hause B, Hajirezaei MR, Druege U. Dark exposure of petunia cuttings strongly improves adventitious root formation and enhances carbohydrate availability during rooting in the light. J Plant Physiol. 2010; doi:10.1016/j.jplph.2009.11.008
32. Hajirezaei M, Sonnewald U, Viola R, Carlisle S, Dennis D, Stitt M. Transgenic potato plants with strongly decreased expression of pyrophosphate:fructose-6-phosphate phosphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. Planta. 1993; doi:10.1007/BF00198688
33. Ahkami AH, Melzer M, Ghaffari MR, Pollmann S, Ghorbani Javid M, Shahinnia F, et al. Distribution of indole-3-acetic acid in *Petunia hybrida* shoot tip cuttings and relationship between auxin transport, carbohydrate metabolism and adventitious root formation. Planta. 2013; doi:10.1007/s00425-013-1907-z
34. Rasmussen A, Hosseini SA, Hajirezaei MR, Druege U, Geelen D. Adventitious rooting declines with the vegetative to reproductive switch and involves a changed auxin homeostasis. J Exp Bot. 2015; doi:10.1093/jxb/eru499
35. Ahkami A, Scholz U, Steuernagel B, Strickert M, Haensch K-T, Druege U, et al. Comprehensive transcriptome analysis unravels the existence of crucial genes regulating primary metabolism during adventitious root formation in *Petunia hybrida*. PLoS One. 2014; doi:10.1371/journal.pone.0100997
36. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003;19:185–93.
37. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett. 2004; doi:10.1016/j.febslet.2004.07.055
38. Laing E, Smith CP. RankProdIt: A web-interactive Rank Products analysis tool. BMC Res Notes. 2010; doi:10.1186/1756-0500-3-221
39. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics. 2002; doi:10.1093/bioinformatics/18.1.207
40. Ott T, Clarke J, Birks K, Johnson G. Regulation of the photosynthetic electron transport chain. Planta. 1999; doi:10.1007/s004250050629
41. Wang D, Portis AR. A novel nucleus-encoded chloroplast protein, PIFI, is involved in NAD(P)H dehydrogenase complex-mediated chlororespiratory electron transport in *Arabidopsis*. Plant Physiol. 2007; doi:10.1104/pp.107.103218

42. Barrett JE, Bartuska CA. S-ABA delays wiltening in petunia and impatiens. Acta Hortic. 2011; doi:10.17660/ActaHortic.2011.893.149
43. Blanchard MG, Newton LA, Runkle ES, Woolard D, Campbell CA. Exogenous application of abscisic acid improved the postharvest drought tolerance of several annual bedding plants. Acta Hortic. 2007; doi:10.17660/ActaHortic.2007.755.16
44. Waterland NL, Campbell CA, Finer JJ, Jones ML. Abscisic acid application enhances drought stress tolerance in bedding plants. HortScience. 2010;45: 409–413.
45. Creelman RA, Bell E, Mullet JE. Involvement of a lipoxygenase-like enzyme in abscisic Acid biosynthesis. Plant Physiol. 1992; doi:10.1104/pp.99.3.1258
46. Han S-Y, Kitahata N, Sekimata K, Saito T, Kobayashi M, Nakashima K, et al. A novel inhibitor of 9-cis-epoxycarotenoid dioxygenase in abscisic acid biosynthesis in higher plants. Plant Physiol. 2004; doi:10.1104/pp.104.039511
47. Ren H, Gao Z, Chen L, Wei K, Liu J, Fan Y, et al. Dynamic analysis of ABA accumulation in relation to the rate of ABA catabolism in maize organs under water deficit. J Exp Bot. 2007; doi:10.1093/jxb/erl117
48. Zhang T, Zhao X, Wang W, Pan Y, Huang L, Liu X, et al. Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes. PLoS One. 2012; doi:10.1371/journal.pone.0043274
49. Stitt M, Hurry V. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr Opin Plant Biol. 2002; doi:10.1016/S1369-5266(02)00258-3
50. Berry J, Bjorkman O. Photosynthetic response and adaptation to temperature in higher plants. Annu Rev Plant Physiol. 1980; doi:10.1146/annurev.pp.31.060180.002423
51. Klopotek Y, George E, Druege U, Klaering H-P. Carbon assimilation of petunia cuttings in a non-disturbed rooting environment: Response to environmental key factors and adventitious root formation. Sci Hortic (Amsterdam). 2012; doi:10.1016/j.scienta.2012.08.004
52. Athanasiou K, Dyson BC, Webster RE, Johnson GN. Dynamic acclimation of photosynthesis increases plant fitness in changing environments. Plant Physiol. 2010; doi:10.1104/pp.109.149351
53. Tallman G. Are diurnal patterns of stomatal movement the result of alternating metabolism of endogenous guard cell ABA and accumulation of ABA delivered to the apoplast around guard cells by transpiration? J Exp Bot. 2004; doi:10.1093/jxb/erh212
54. Bihmidine S, Hunter CT, Johns CE, Koch KE, Braun DM. Regulation of assimilate import into sink organs: update on molecular drivers of sink strength. Front Plant Sci. 2013; doi:10.3389/fpls.2013.00177
55. Ahkami AH, Lischewski S, Haensch K-T, Porfirova S, Hofmann J, Rolletschek H, et al. Molecular physiology of adventitious root formation in *Petunia hybrida* cuttings: involvement

of wound response and primary metabolism. New Phytol. 2009; doi:10.1111/j.1469-8137.2008.02704.x

56. Klopotek Y, Franken P, Klaering H-P, Fischer K, Hause B, Hajirezaei M-R, et al. A higher sink competitiveness of the rooting zone and invertases are involved in dark stimulation of adventitious root formation in *Petunia hybrida* cuttings. Plant Sci. 2016; doi:10.1016/j.plantsci.2015.11.001
57. Sturm A. Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. PLANT Physiol. 1999; doi:10.1104/pp.121.1.1
58. Gibeaut DM, Karuppiah N, Chang S-R, Brock TG, Vadlamudi B, Kim D, et al. Cell wall and enzyme changes during the graviresponse of the leaf-sheath pulvinus of oat (*Avena sativa*). Plant Physiol. 1990; doi:10.1104/pp.94.2.411
59. Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo HG. Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. Plant J. 1999; doi:10.1046/j.1365-313X.1999.00609.x
60. Saze H, Ueno Y, Hisabori T, Hayashi H, Izui K. Thioredoxin-mediated reductive activation of a protein kinase for the regulatory phosphorylation of C4-form phosphoenolpyruvate carboxylase from maize. Plant Cell Physiol. 2001; doi:10.1093/pcp/pce182
61. Cousins AB, Baroli I, Badger MR, Ivakov A, Lea PJ, Leegood RC, et al. The role of phosphoenolpyruvate carboxylase during C_4 photosynthetic isotope exchange and stomatal conductance. Plant Physiol. 2007; doi:10.1104/pp.107.103390
62. Vaasen A, Bergerow D, Hampp R. Phosphoenolpyruvate carboxylase genes in C_3, crassulacean acid metabolism (CAM) and C_3/CAM intermediate species of the genus Clusia: rapid reversible C_3/CAM switches are based on the C_3 housekeeping gene. Plant, Cell Environ. 2006; doi:10.1111/j.1365-3040.2006.01583.x
63. Shibasaki K, Uemura M, Tsurumi S, Rahman A. Auxin response in *Arabidopsis* under cold stress: underlying molecular mechanisms. Plant Cell. 2009; doi:10.1105/tpc.109.069906
64. Bajguz A, Piotrowska A. Conjugates of auxin and cytokinin. Phytochemistry. 2009; doi:10.1016/j.phytochem.2009.05.006
65. Ren H, Gray WM. SAUR proteins as effectors of hormonal and environmental signals in plant growth. Mol Plant. Elsevier Ltd; 2015; doi:10.1016/j.molp.2015.05.003
66. Stavang JA, Lindgård B, Erntsen A, Lid SE, Moe R, Olsen JE. Thermoperiodic stem elongation involves transcriptional regulation of gibberellin deactivation in pea. Plant Physiol. 2005; doi:10.1104/pp.105.063149
67. Stavang JA, Gallego-Bartolomé J, Gómez MD, Yoshida S, Asami T, Olsen JE, et al. Hormonal regulation of temperature-induced growth in *Arabidopsis*. Plant J. 2009; doi:10.1111/j.1365-313X.2009.03983.x
68. Stavang JA, Pettersen RI, Wendell M, Solhaug KA, Junttila O, Moe R, et al. Thermoperiodic

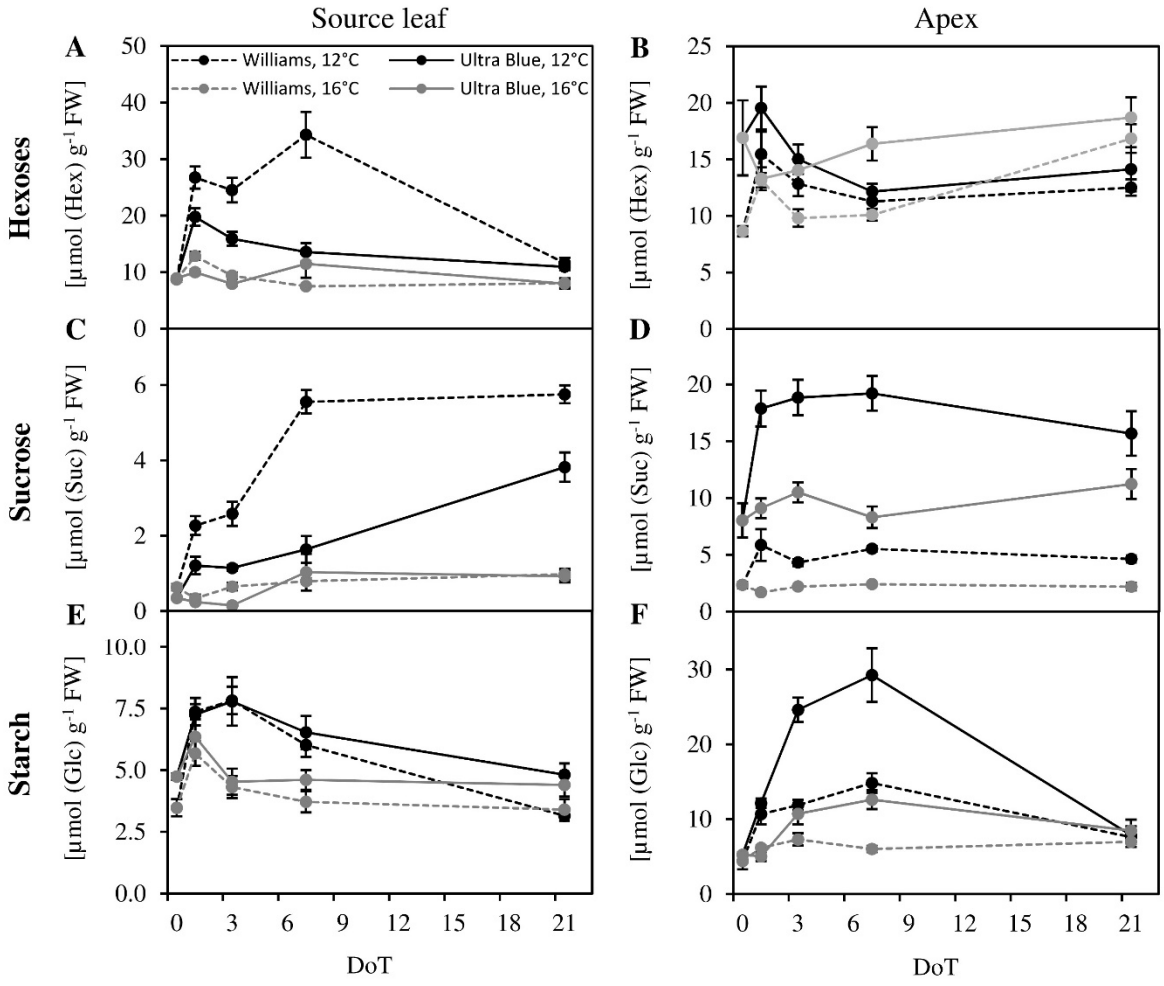
growth control by gibberellin does not involve changes in photosynthetic or respiratory capacities in pea. J Exp Bot. 2010; doi:10.1093/jxb/erp366

69. Topp SH, Rasmussen SK, Sander L. Alcohol induced silencing of gibberellin 20-oxidases in *Kalanchoe blossfeldiana*. Plant Cell Organ Organ Cult. 2008; doi:10.1007/s11240-008-9368-y
70. Sun T-p. Gibberellin-GID1-DELLA: A Pivotal Regulatory Module for Plant Growth and Development. Plant Physiol. 2010; doi:10.1104/pp.110.161554
71. Davière J-M, Achard P. Gibberellin signaling in plants. Development. 2013; doi:10.1242/dev.087650
72. Yang Y-W, Chen H-C, Jen W-F, Liu L-Y, Chang M-C. Comparative transcriptome analysis of shoots and roots of TNG67 and TCN1 rice seedlings under cold stress and following subsequent recovery: insights into metabolic pathways, phytohormones, and transcription factors. PLoS One. 2015; doi:10.1371/journal.pone.0131391
73. Mega R, Meguro-Maoka A, Endo A, Shimosaka E, Murayama S, Nambara E, et al. Sustained low abscisic acid levels increase seedling vigor under cold stress in rice (*Oryza sativa L.*). Sci Rep. 2015; doi:10.1038/srep13819
74. Nambara E, Marion-Poll A. Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol. 2005; doi:10.1146/annurev.arplant.56.032604.144046
75. Golan I, Dominguez PG, Konrad Z, Shkolnik-Inbar D, Carrari F, Bar-Zvi D. Tomato *ABSCISIC ACID STRESS RIPENING (ASR)* gene family revisited. PLoS One. 2014; doi:10.1371/journal.pone.0107117
76. Kim IS, Kim YS, Yoon HS. Rice ASR1 protein with reactive oxygen species scavenging and chaperone-like activities enhances acquired tolerance to abiotic stresses in *Saccharomyces cerevisiae*. Mol Cells. 2012; doi:10.1007/s10059-012-2253-x
77. Saumonneau A, Agasse A, Bidoyen MT, Lallemand M, Cantereau A, Medici A, et al. Interaction of grape ASR proteins with a DREB transcription factor in the nucleus. FEBS Lett. 2008; doi:10.1016/j.febslet.2008.09.015
78. Ren XD, Liu HM, Liu YH, Hu YF, Zhang JJ, Huang YB. Influence of sugars and hormones on the genes involved in sucrose metabolism in maize endosperms. Genet Mol Res. 2015; doi:10.4238/2015.March.6.13
79. Chen J, Huang B, Li Y, Du H, Gu Y, Liu H, et al. Synergistic influence of sucrose and abscisic acid on the genes involved in starch synthesis in maize endosperm. Carbohydr Res. 2011; doi:10.1016/j.carres.2011.05.003
80. Trouverie J, Thevenot C, Rocher J-P, Sotta B, Prioul J-L. The role of abscisic acid in the response of a specific vacuolar invertase to water stress in the adult maize leaf. J Exp Bot. 2003; doi:10.1093/jxb/erg234
81. Trouverie J, Chateau-Joubert S, Thévenot C, Jacquemot M-P, Prioul J-L. Regulation of vacuolar invertase by abscisic acid or glucose in leaves and roots from maize plantlets. Planta.

2004; doi:10.1007/s00425-004-1289-3

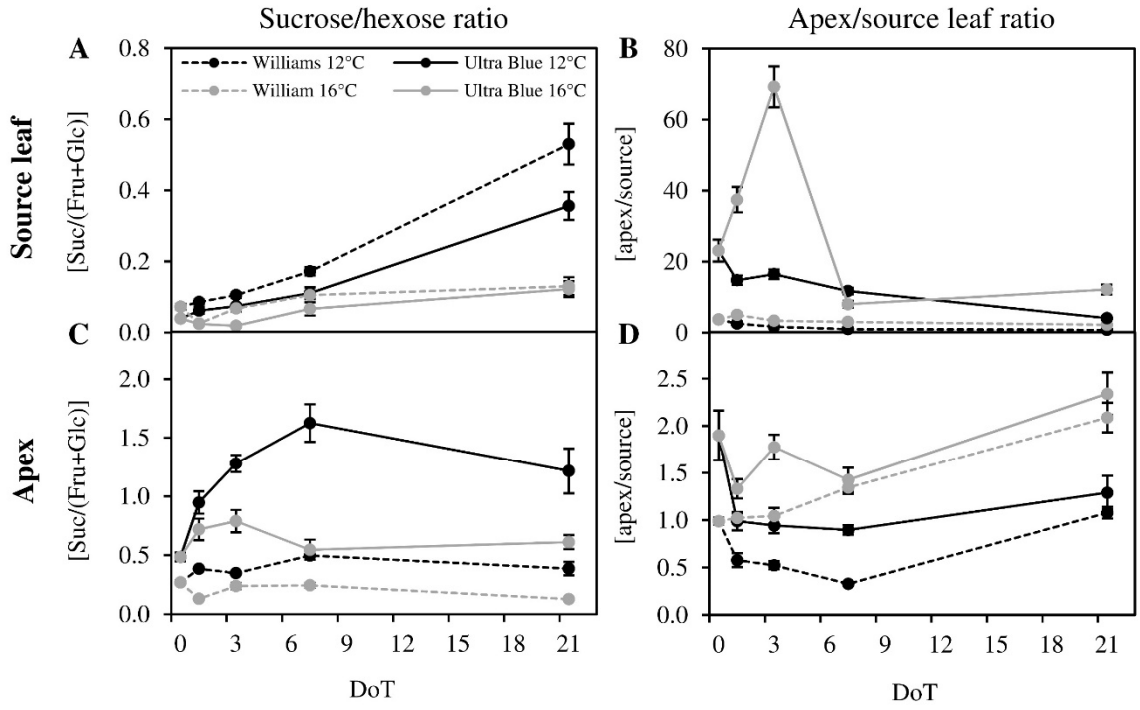
82. LeNoble ME. Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. J Exp Bot. 2003;55:237–245. doi:10.1093/jxb/erh031
83. Spollen WG, LeNoble ME, Samuels TD, Bernstein N, Sharp RE. Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. Plant Physiol. 2000; doi:10.1104/pp.122.3.967
84. Shi Y, Tian S, Hou L, Huang X, Zhang X, Guo H, et al. Ethylene signaling negatively regulates freezing tolerance by repressing expression of *CBF* and type-A *ARR* genes in *Arabidopsis*. Plant Cell. 2012; doi:10.1105/tpc.112.098640
85. Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A. Contrapuntal role of ABA: Does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene. 2012; doi:10.1016/j.gene.2012.06.076
86. Jiang W, Bai J, Yang X, Yu H, Liu Y. Exogenous application of abscisic acid, putrescine, or 2,4-epibrassinolide at appropriate oncentrations effectively alleviate damage to tomato seedlings from suboptimal temperature stress. Horttechnology. 2012;1: 137–144.
87. Shinkawa R, Morishita A, Amikura K, Machida R, Murakawa H, Kuchitsu K, et al. Abscisic acid induced freezing tolerance in chilling-sensitive suspension cultures and seedlings of rice. BMC Res Notes. 2013; doi:10.1186/1756-0500-6-351
88. Drüge U, Schönbeck F. Effect of vesicular-arbuscular mycorrhizal infection on transpiration, photosynthesis and growth of flax (*Linum usitatissimum L.*) in relation to cytokinin levels. Journal of Plant Physiology. 1993. doi:10.1016/S0176-1617(11)80849-7
89. Meguro A, Sato Y. Salicylic acid antagonizes abscisic acid inhibition of shoot growth and cell cycle progression in rice. Sci Rep. 2014; doi:10.1038/srep04555
90. Estrada-Melo AC, Chao, Reid MS, Jiang C-Z. Overexpression of an ABA biosynthesis gene using a stress-inducible promoter enhances drought resistance in petunia. Hortic Res. 2015; doi:10.1038/hortres.2015.13
91. Wang L, Zhao C-M, Wang Y-J, Liu J. Overexpression of chloroplast-localized small molecular heat-shock protein enhances chilling tolerance in tomato plant. Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao. 2005;31: 167–74.

Figure legends



		Source leaf					Apex				
		DoT					DoT				
		0	1	3	7	21	0	1	3	7	21
Hexoses	C	n.s.	**	**	*	n.s.	*	n.s.	*	**	n.s.
	T	-	***	***	***	**	-	*	n.s.	n.s.	*
	C x T	-	n.s.	*	***	n.s.	-	n.s.	n.s.	*	n.s.
Sucrose	C	*	*	***	***	**	**	***	***	***	***
	T	-	***	***	***	***	-	***	***	***	*
	C x T	-	*	*	***	**	-	n.s.	*	**	n.s.
Starch	C	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	***	***	n.s.
	T	-	*	***	**	n.s..	-	***	***	***	n.s.
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	**	n.s.	n.s.

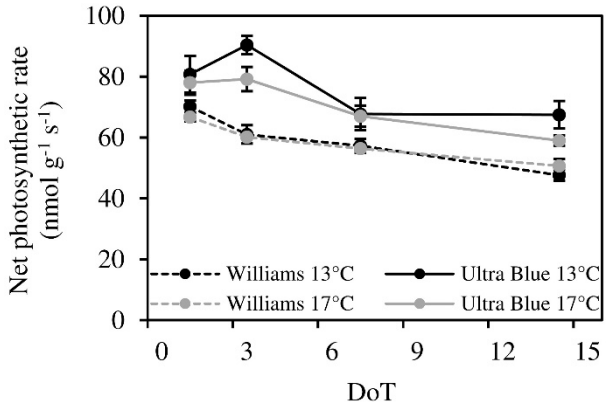
Fig. 1 Carbohydrate concentrations. Impact of chilling on carbohydrate concentrations (straight lines: UB, dotted lines: SW; black: 12 °C, grey: 16 °C). (A) source leaf, hexoses; (B) apex, hexoses; (C) source leaf, sucrose; (D) apex, sucrose; (E) source leaf, starch (specified in units of Glc); (F) apex, starch (specified in units of Glc). (DoT = days after differentiation of temperature; n = 9; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$; n.s. = not significant).



		DoT				
		0	1	3	7	21
Suc/hexose (source leaf)	C	*	n.s.	***	*	*
	T	-	***	***	**	***
	C x T	-	n.s.	n.s.	n.s.	*
Suc/hexose (apex)	C	**	***	***	***	***
	T	-	*	***	***	**
	C x T	-	n.s.	*	**	n.s.

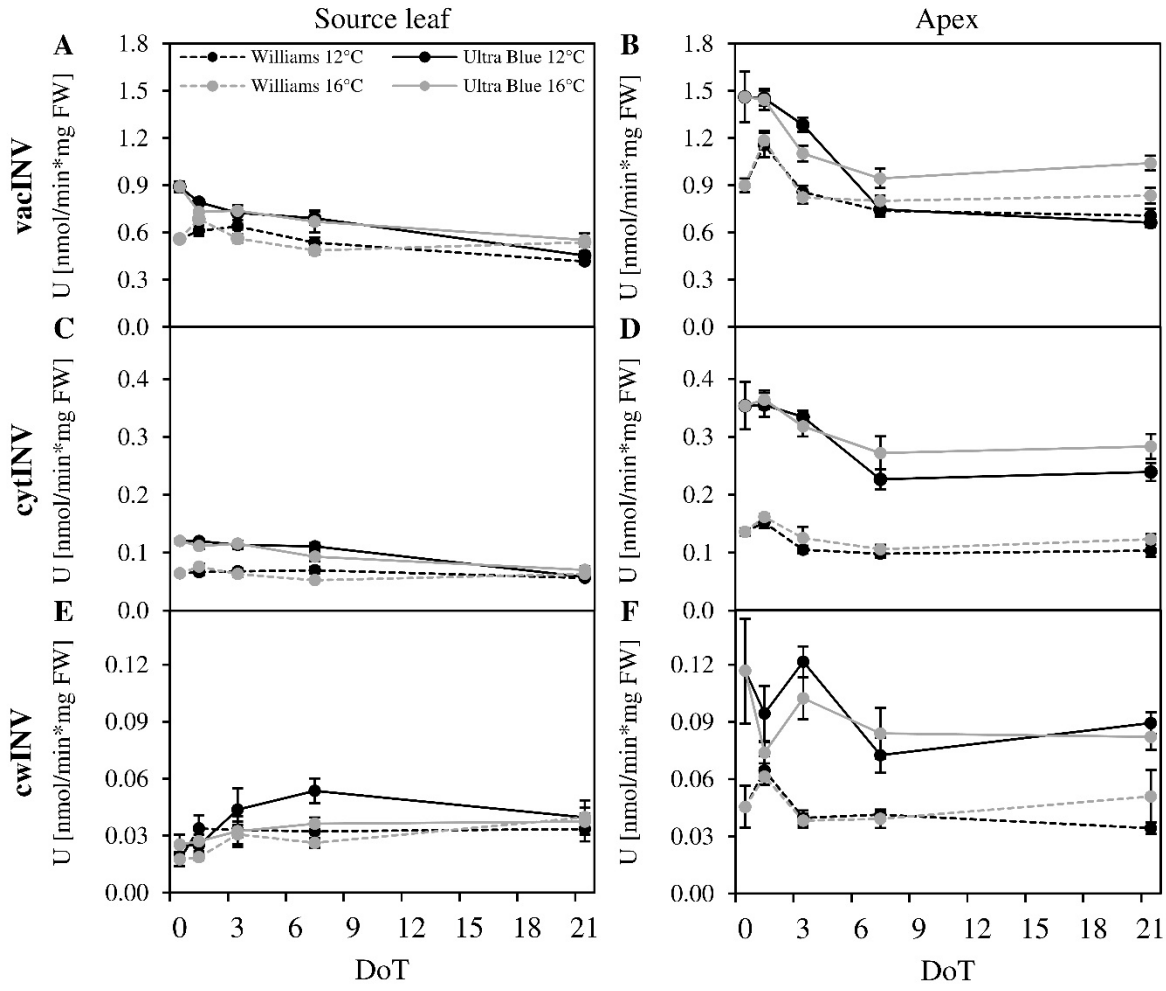
	DoT				
	0	1	3	7	21
Apex/source leaf (sucrose)	***	***	***	***	***
	-	***	***	n.s.	***
	-	***	***	**	**
Apex/source leaf (hexoses)	*	**	***	**	n.s.
	-	***	***	***	***
	-	n.s.	n.s.	*	n.s.

Fig. 2 Carbohydrate ratios. Impact of chilling on carbohydrate ratios between sucrose (Suc) and hexoses (glucose: Glc, and fructose: Fru) respectively between the apex and the source leaf (straight lines: UB, dotted lines: SW; black: 12 °C, grey: 16 °C). (A) source leaf, Suc:hexose ratio; (B) Suc, apex:source leaf ratio; (C) apex, Suc:hexose ratio; (D) hexoses, apex:source leaf ratio. (DoT = days after differentiation of temperature; n = 9; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$; n.s. = not significant).



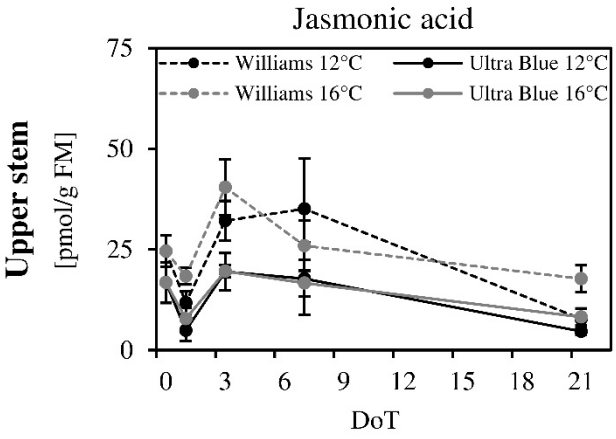
		DoT			
		1	3	7	14
Net photo-synthetic rate	C	*	***	*	**
	T	n.s.	n.s.	n.s.	n.s.
	C x T	n.s.	n.s.	n.s.	n.s.

Fig. 3 Net photosynthetic rate. Impact of chilling on net photosynthetic rates on leaf dry weight basis (straight lines: UB, dotted lines: SW; black: 13 °C, grey: 17 °C). (photosynthetically active radiation [150 µmol m^{-2} s^{-1}]; DoT = days after differentiation of temperature; n = 4; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$; n.s. = not significant).



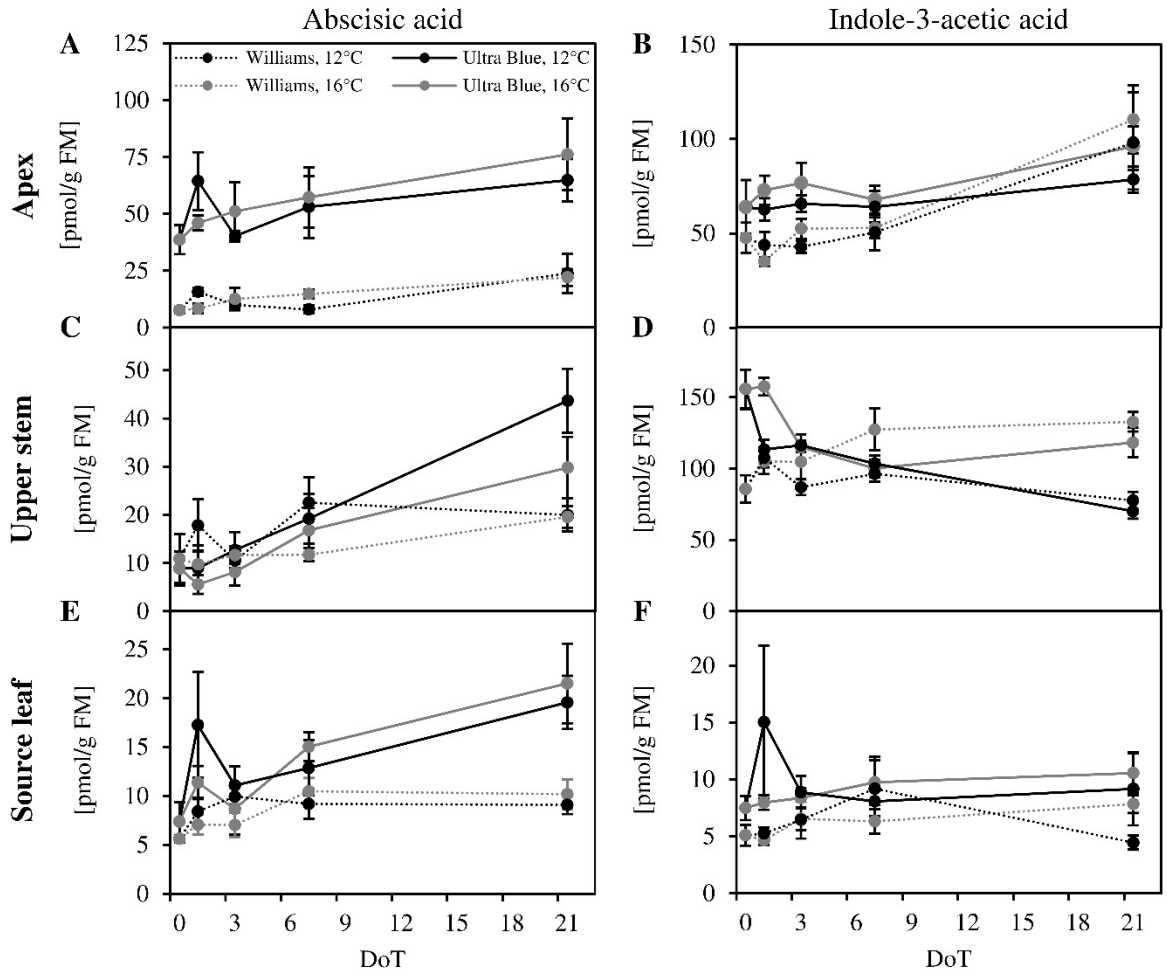
		Source leaf					Apex				
		DoT					DoT				
		0	1	3	7	21	0	1	3	7	21
vacINV	C	***	*	***	**	n.s.	**	**	***	n.s.	n.s.
	T	-	n.s.	n.s.	n.s.	**	-	n.s.	*	*	***
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	*
cytINV	C	***	***	***	***	n.s.	***	***	***	***	***
	T	-	n.s.	n.s.	*	*	-	n.s.	n.s.	n.s.	*
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.
cwINV	C	n.s.	n.s.	n.s.	**	n.s.	*	*	***	**	***
	T	-	n.s.	n.s.	*	n.s.	-	n.s.	n.s.	n.s.	n.s.
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.

Fig. 4 Invertase activities. Impact of chilling on the activities of vacuolar invertase (vacINV), cytosolic invertase (cytINV) and cell wall invertase (cwINV) (straight lines: UB, dotted lines: SW; black: 12 °C, grey: 16 °C). (A) source leaf, vacINV; (B) apex, vacINV; (C) source leaf, cytINV; (D) apex, cytINV; (E) source leaf, cwINV; (F) apex, cwINV. (DoT = days after differentiation of temperature; n = 9; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$; n.s. = not significant).



		DoT				
		0	1	3	7	21
Upper stem	C	n.s.	*	*	n.s.	*
	T	-	n.s.	n.s.	n.s.	*
	C x T	-	n.s.	n.s.	n.s.	n.s.

Fig. 5 Concentrations of jasmonic acid. Impact of chilling on levels of jasmonic acid in the upper stem (straight lines: UB, dotted lines: SW; black: 12 °C, grey: 16 °C). (DoT = days after differentiation of temperature; n = 6; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, n.s. = not significant).



		Abscisic acid					Indole-3-acetic acid				
		DoT					DoT				
		0	1	3	7	21	0	1	3	7	21
Apex	C	***	***	***	**	***	n.s.	**	*	n.s.	n.s.
	T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.
Upper stem	C	n.s.	n.s.	n.s.	n.s.	*	***	**	*	n.s.	n.s.
	T	-	n.s.	n.s.	n.s.	n.s.	-	*	n.s.	n.s.	***
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	*	n.s.	n.s.	n.s.
Source leaf	C	n.s.	*	n.s.	*	**	*	n.s.	n.s.	n.s.	n.s.
	T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.
	C x T	-	n.s.	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	n.s.

Fig. 6 Concentrations of abscisic acid and indole-3-acetic acid. Impact of chilling on levels of abscisic acid (ABA) and indole-3-acetic acid (IAA) (straight lines: UB, dotted lines: SW; black: 12 °C, grey: 16 °C). (A) apex, ABA; (B) apex, IAA; (C) upper stem, ABA; (D) upper stem, IAA; (E) source leaf, ABA; (F) source leaf, IAA. (DoT = days after differentiation of temperature; $n = 6$; In the Significance Table, asterisks indicate significance levels of the effects of cultivar (C) and temperature (T) and of interactions between C and T for the specified DoT, * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$; n.s. = not significant).

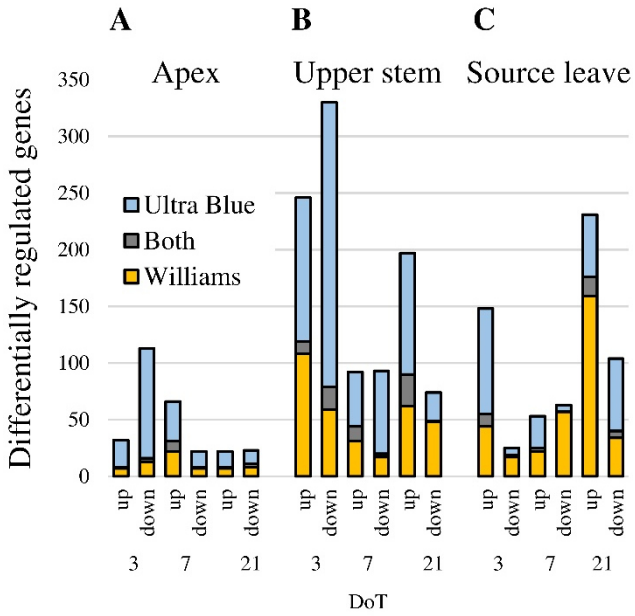


Fig. 7 Numbers of chilling responsive genes in dependence on cultivar. Numbers of annotated genes, which are up- respectively down-regulated under the impact of chilling compared to control. (A) apex; (B) upper stem; (C) source leaf. (Gene expression was analyzed at 3, 7 and 21 DoT: yellow and blue colors indicate genes regulated differentially only in SW, or only in UB, respectively, grew color indicates genes, differentially regulated in both cultivars; DoT = days after differentiation of temperature; up-regulated: log2 > 1; down-regulated: log2 < -1; pfp-value < 0.15).

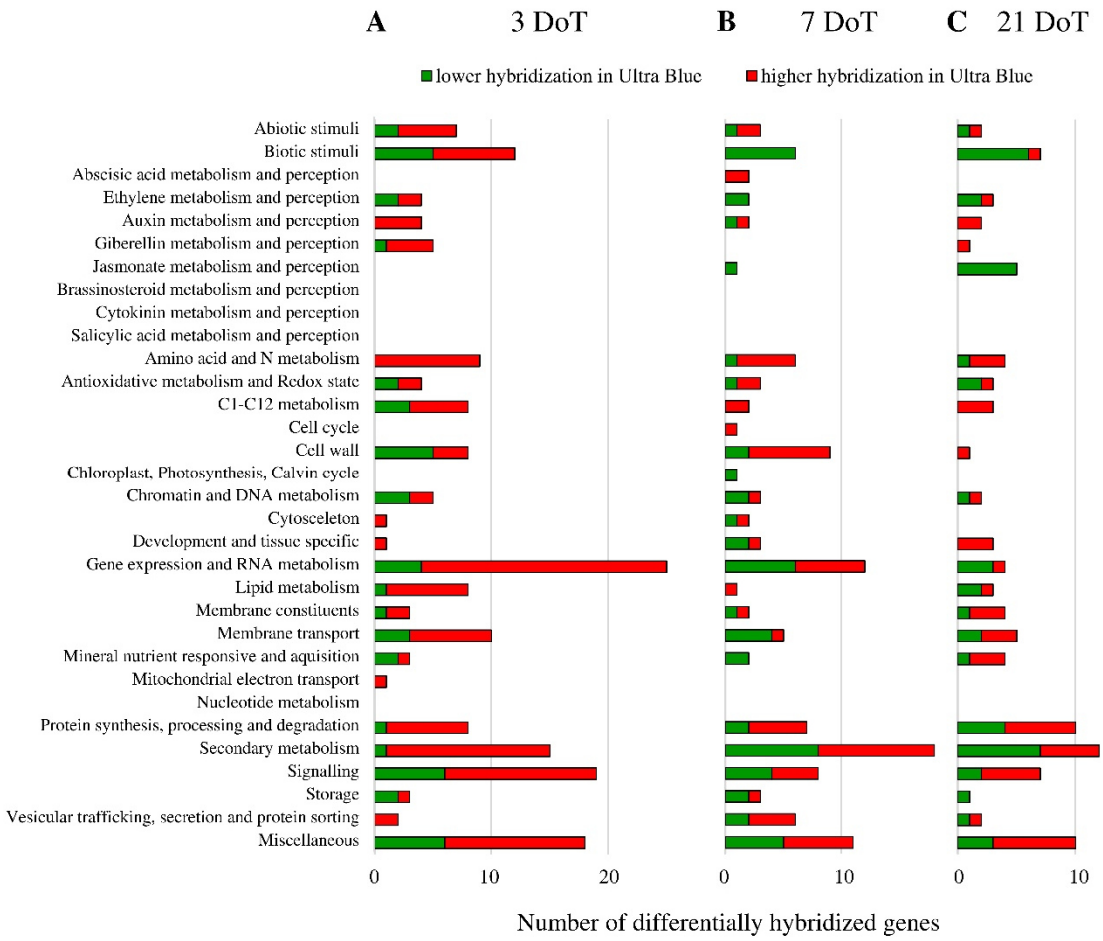


Fig. 8 Numbers and functions of chilling-dependent cultivar-specifically regulated genes in the apex. Numbers of annotated genes, which were differentially expressed in UB and SW only at chilling and not at control temperature in the apex. (A) 3 DoT; (B) 7 DoT; (C) 21 DoT. Red color indicates genes with significantly higher expression rates in UB, green color indicates lower expression rates in UB. (DoT = days after differentiation of temperature; higher expression rates in UB: log2 > 1; lower expression rates in UB: log2 < -1; pfp-value < 0.15).

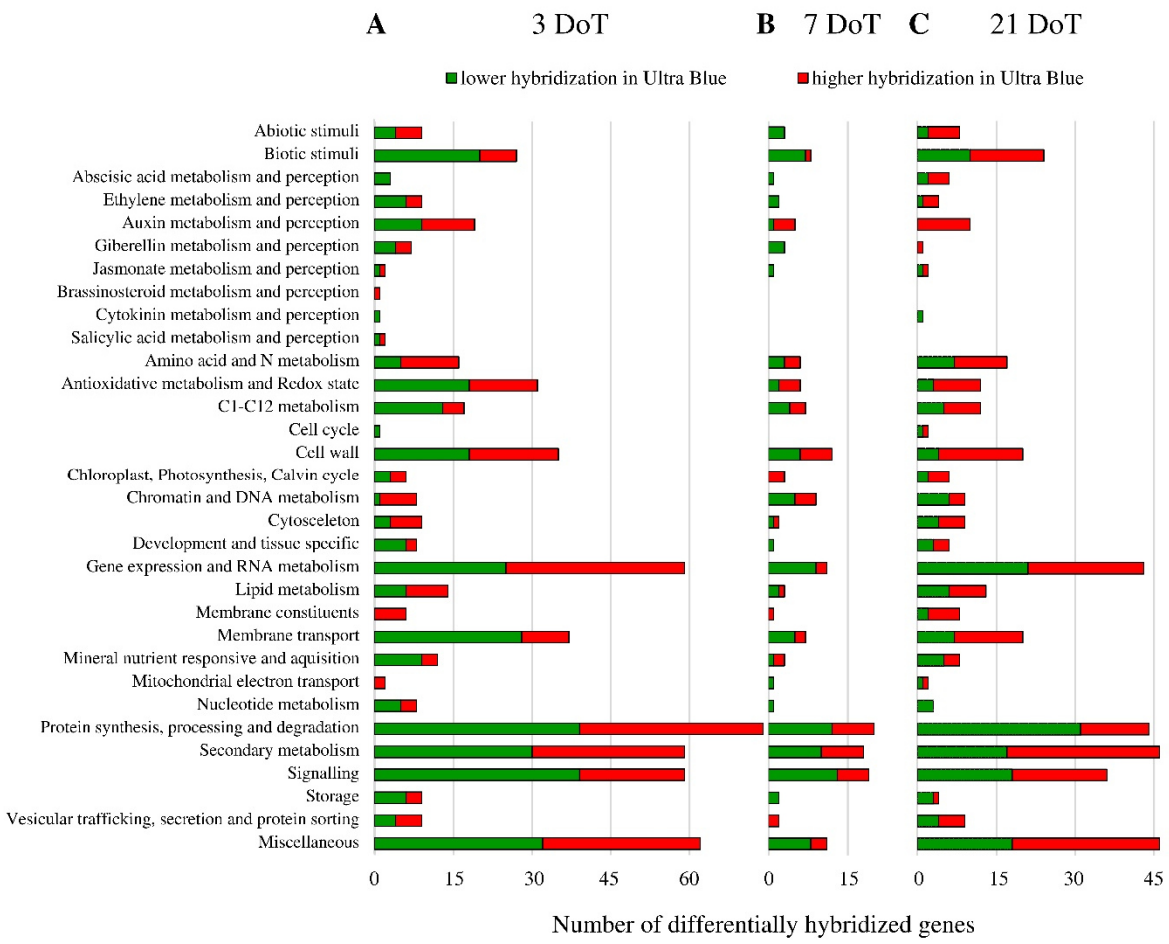


Fig. 9 Numbers and functions of chilling-dependent cultivar-specifically regulated genes in the upper stem. Numbers of annotated genes, which were differentially expressed in UB and SW only at chilling and not at control temperature in the upper stem. (A) 3 DoT; (B) 7 DoT; (C) 21 DoT. Red color indicates genes with significantly higher expression rates in UB, green color indicates lower expression rates in UB. (DoT = days after differentiation of temperature; higher expression rates in UB: log2 > 1; lower expression rates in UB: log2 < -1; pfp-value < 0.15).

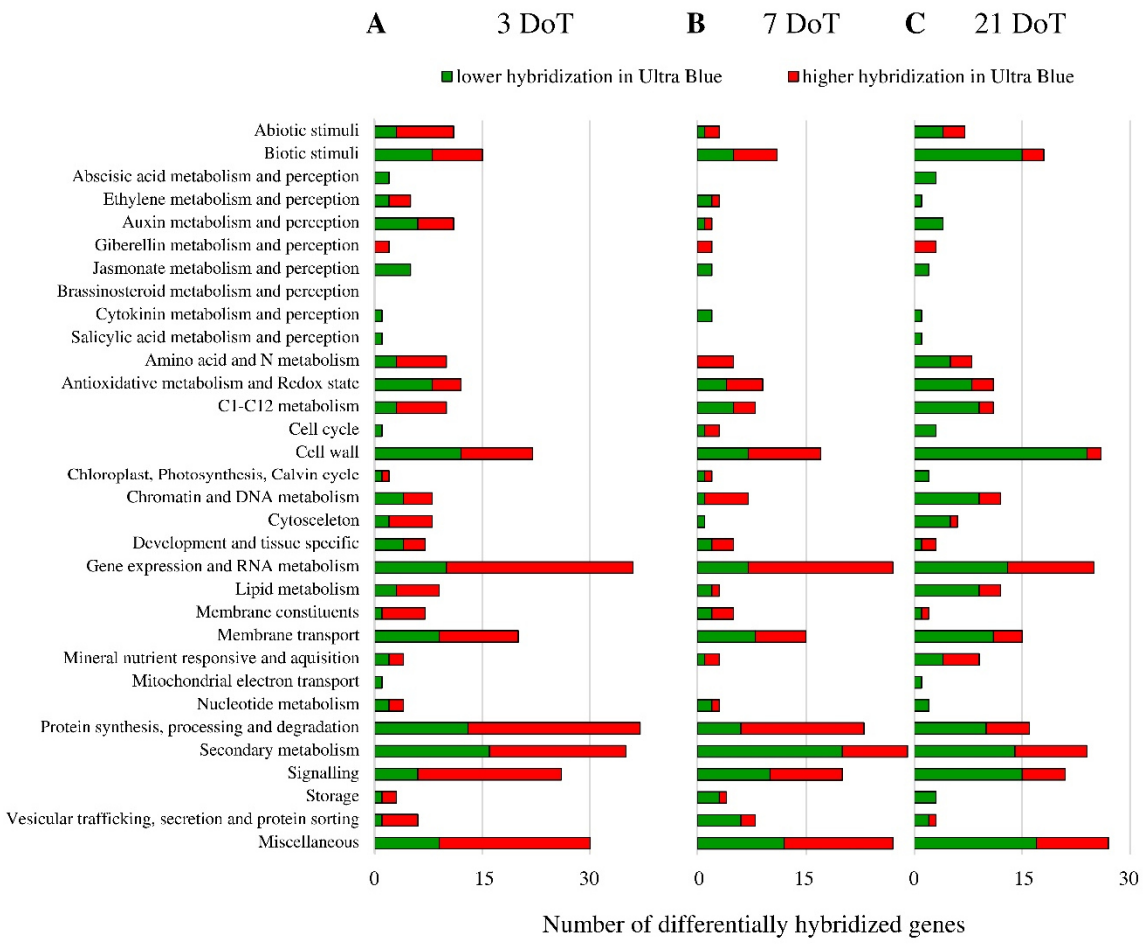


Fig. 10 Numbers and functions of chilling-dependent cultivar-specifically regulated genes in the source leaf. Numbers of annotated genes, which were differentially expressed in UB and SW only at chilling and not at control temperature in the source leaf. (A) 3 DoT; (B) 7 DoT; (C) 21 DoT. Red color indicates genes with significantly higher expression rates in UB, green color indicates lower expression rates in UB. (DoT = days after differentiation of temperature; higher expression rates in UB: log2 > 1; lower expression rates in UB: log2 < -1; pfp-value < 0.15).

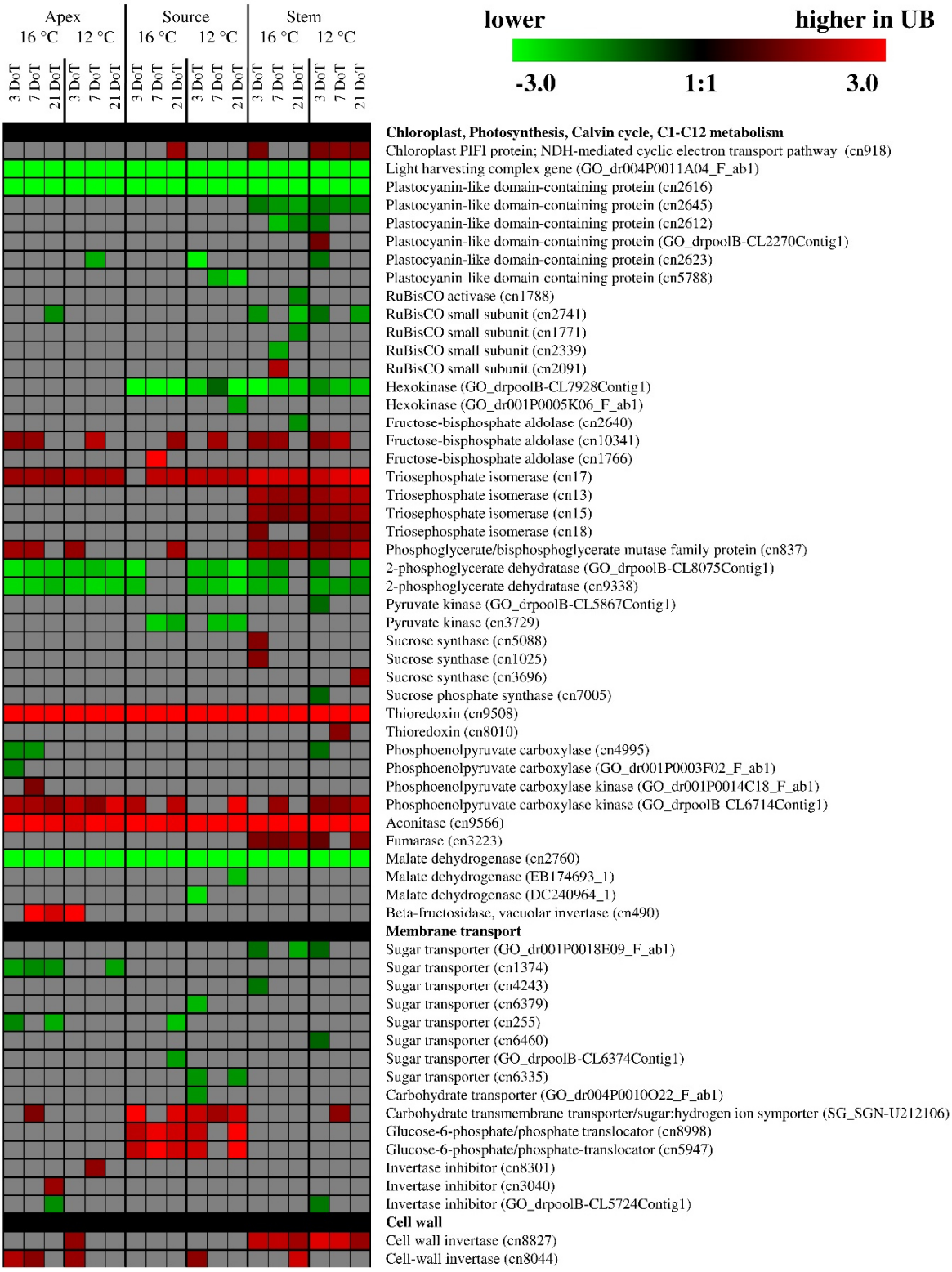
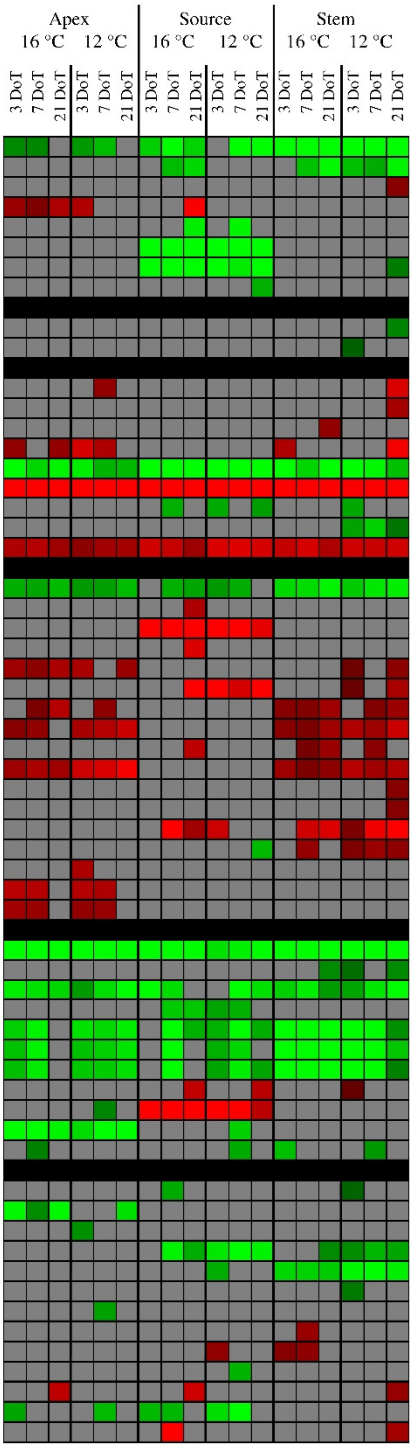
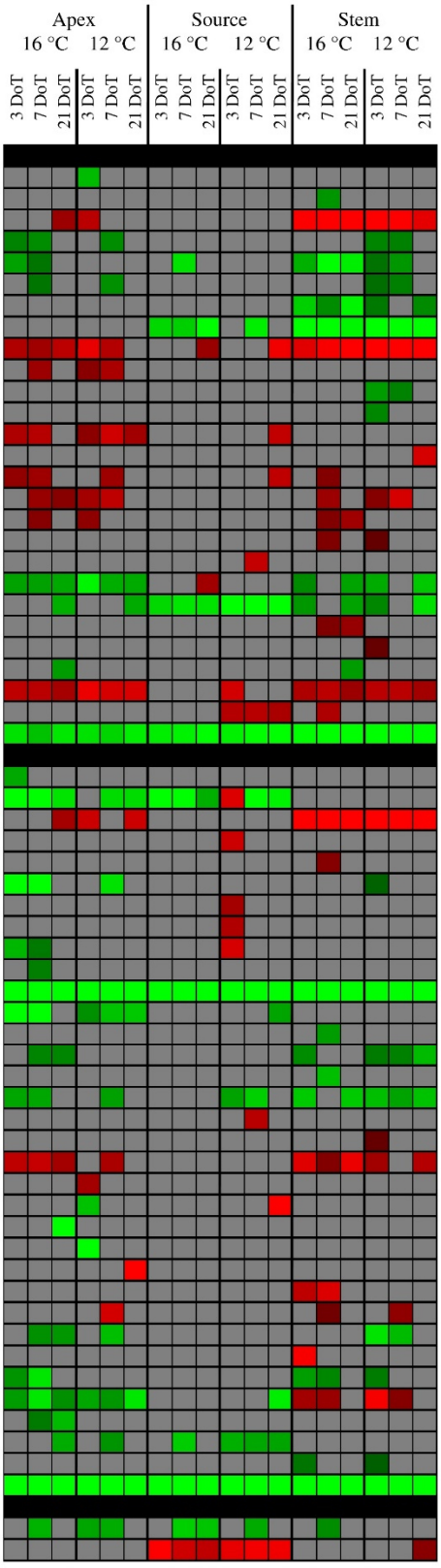


Fig. 11 Selection of relevant genes cultivar-specifically expressed under control or chilling conditions. Selection of relevant genes that are putatively involved in the observed metabolic changes and in plant hormone-related pathways (for the complete list of differentially expressed genes, see Additional file 5: Table S1C). Cultivar-specific differences are shown for the apex, the source leaf and the stem at 3, 7 and 21 DoT for 12 °C and 16 °C. Colored squares represent Log2 values of differences in the expression rates between both cultivars for individual genes: red color represents genes, which showed higher expression rates in UB, green color represents genes, which showed lower expression rates in UB; the intensity of the colors indicate the extend of expression differences (pfp-value < 0.15).



Invertase/pectin methylesterase inhibitor family protein (GO_dr004P0010F08_F_ab1)
Invertase/pectin methylesterase inhibitor family protein (cn3181)
Invertase/pectin methylesterase inhibitor family protein (GO_dr004P0003P14_F_ab1)
Invertase/pectin methylesterase inhibitor family protein (cn7252)
Invertase/pectin methylesterase inhibitor family protein (GO_dr004P0015G17_F_ab1)
Invertase/pectin methylesterase inhibitor family protein (GO_dr001P0001J21_F_ab1)
Invertase/pectin methylesterase inhibitor family protein (GO_drpoolB-CL4433Contig1)
Invertase/pectin methylesterase inhibitor family protein (cn5964)
Storage
Starch synthase (GO_dr004P0011A22_F_ab1)
Starch synthase (SG_SGN-U211286)
Abscisic acid metabolism and perception
9-cis-epoxy-carotenoid dioxygenase 1 (cn9067)
9-cis-epoxy-carotenoid dioxygenase 1 (cn9068)
9-cis-epoxy-carotenoid dioxygenase 2 (GO_drpoolB-CL6861Contig1)
9-cis-epoxy-carotenoid dioxygenase 3 (GO_drpoolB-CL9680Contig1)
Abscisic stress ripening protein (cn8)
Abscisic stress ripening protein (cn127)
Abscisic stress ripening protein (cn6)
Abscisic stress ripening protein (cn9885)
Carotenoid cleavage dioxygenase (cn3078)
Auxin metabolism and perception
Aux/IAA protein (cn368)
Aux/IAA protein (cn369)
Auxin efflux carrier (GO_dr004P0031O04_F_ab1)
Auxin efflux carrier family protein (GO_dr001P0004B13_F_ab1)
Auxin transport facilitator protein (IP_PHBS012P22u)
Auxin:hydrogen symporter (cn3141)
Auxin-induced SAUR-like protein (IP_PHBS002A17u)
Auxin-induced SAUR-like protein (cn7023)
Auxin-induced SAUR-like protein (cn7640)
Auxin-induced SAUR-like protein (cn9969)
Auxin-induced SAUR-like protein (GO_drpoolB-CL7626Contig1)
Auxin-induced SAUR-like protein (cn7444)
SAUR family protein (GO_drpoolB-CL4485Contig1)
SAUR family protein (GO_dr004P0015K14_F_ab1)
SAUR family protein (GO_dr001P0010E10_F_ab1)
IAA amidohydrolase (IP_PHBS003G07u)
IAA-amino acid hydrolase (GO_dr001P0019G03_F_ab1)
Jasmonate metabolism and perception
12-oxophytodienoate reductase (GO_drpoolB-CL7989Contig1)
12-oxophytodienoate reductase (cn4400)
Beta-hydroxyacyl-ACP dehydratase (cn9201)
Chloroplast allene oxide synthase (cn9035)
Lipoxygenase (cn1171)
Lipoxygenase (SG_SGN-U211560)
Lipoxygenase (EB174935_1)
Lipoxygenase (GO_drpoolB-CL2440Contig1)
Lipoxygenase (GO_drpoolB-CL3847Contig1)
Lipoxygenase (cn9038)
Lipoxygenase (cn729)
Ethylene metabolism and perception
1-aminocyclopropane-1-carboxylate oxidase (cn1774)
1-aminocyclopropane-1-carboxylate oxidase (cn3506)
1-aminocyclopropane-1-carboxylate oxidase (cn1898)
1-aminocyclopropane-1-carboxylate oxidase (IP_PHBS005F17u)
1-aminocyclopropane-1-carboxylic acid oxidase (GO_drpoolB-CL7401Contig1)
1-aminocyclopropane-1-carboxylic acid oxidase (GO_dr001P0020M20_F_ab1)
1-aminocyclopropane-1-carboxylate oxidase 4 (cn2105)
1-aminocyclopropane-1-carboxylate oxidase 4 (cn1901)
1-aminocyclopropane-1-carboxylate oxidase 4 (cn1902)
1-aminocyclopropane-1-carboxylate synthase (cn3690)
1-aminocyclopropane-1-carboxylate synthase (IP_PHBS001D24u)
Aminocyclopropane-1-carboxylate synthase (GO_dr001P0018F20_F_ab1)
Aminocyclopropane-1-carboxylate synthase (cn3385)



Giberellin metabolism and perception
Copalyl diphosphate synthase (cn8481)
Copalyl diphosphate synthase (GO_drpoolB-CL841Contig1)
DELLA protein (GA response repressor) (GO_dr004P0023M03_F_ab1)
Ent-kaurene oxidase (GO_drpoolB-CL177Contig1)
Ent-kaurenoic acid oxidase (cn911)
GA2 ent-kaurene synthase (GO_drpoolB-CL916Contig1)
Gibberellin 20 oxidase (cn6283)
Gibberellin 20 oxidase (cn8430)
Gibberellin 20 oxidase (GO_drpoolB-CL1388Contig1)
Gibberellin 20 oxidase (cn2849)
Gibberellin 20 oxidase (cn292)
Gibberellin 20 oxidase (cn290)
Gibberellin 20 oxidase (cn7812)
Gibberellin 20 oxidase (cn1024)
Gibberellin 20 oxidase (GO_drpoolB-CL9449Contig1)
Gibberellin 2-beta-dioxygenase (cn1090)
Gibberellin 2-oxidase (cn577)
Gibberellin 7-oxidase (GO_dr001P0004N14_F_ab1)
Gibberellin-induced protein (cn832)
Gibberellin-regulated GASA/GAST/Snakin family protein (GO_drpoolB-CL4258Contig1)
Gibberellin-responsive Gip1-like protein (cn3295)
Gibberellin-responsive Gip1-like protein (cn3300)
Gibberellin-responsive Gip1-like protein (cn3301)
Gibberellin receptor GID1 (cn8914)
Gibberellin receptor GID1 (cn6177)
Gibberellin receptor GID1 (GO_dr001P0002O14_F_ab1)
Gibberellin receptor GID2 (GO_drpoolB-CL5608Contig1)
Abiotic stimuli
Chloroplast small heat shock protein (GO_dr001P0001M13_F_ab1)
Chloroplast small heat shock protein class I (DY396161_1)
Mitochondrial small heat shock protein (DY395946_1)
Mitochondrial small heat shock protein (IP_PHBS004A14u)
Mitochondrial small heat shock protein (cn1894)
Class II small heat shock protein (cn1765)
Low molecular weight heat-shock protein (DC241174_1)
Low molecular weight heat-shock protein (DY396020_1)
Low molecular weight heat-shock protein (cn10327)
Low molecular weight heat-shock protein (cn10426)
Low temperature and salt responsive protein (cn3240)
17.5 kDa class II heat shock protein (DY395836_1)
Heat shock 80 kDa protein (cn2534)
Heat shock 81 kDa protein (cn2620)
Heat shock 81 kDa protein (GO_drpoolB-CL8383Contig1)
Heat shock protein (GO_dr001P0012O14_F_ab1)
Heat shock protein (cn2328)
Heat shock protein (GO_dr004P0011J05_F_ab1)
Heat shock protein 70 (cn10275)
Heat shock protein 70 (HSP70)-interacting protein (DY396022_1)
Heat shock protein 81-1 (cn1705)
Heat shock protein 81-2 (cn2527)
Heat shock protein 83 (cn2521)
Heat shock protein 83 (DY395618_1)
Heat shock protein Hsp20 (GO_drpoolB-CL4311Contig1)
Heat shock protein Hsp20 (GO_dr001P0006F02_F_ab1)
Heat-shock protein (cn529)
Heat-shock protein (cn10513)
Heat shock protein 18.2 (cn10425)
Dehydrin (cn859)
Cold regulated 413 plasma membrane 2 (cn1592)
Cold regulated 413 plasma membrane protein (cn7999)
Cold-inducible RNA binding protein (cn901)
Drought-responsive family protein (cn6228)
Gene expression and RNA metabolism
Transcription factor ERF029 (GO_dr004P0029M08_F_ab1)
CCAAT-binding transcription factor CBF-B/NF-YA family protein (GO_drs12P0022D23_F_ab1)

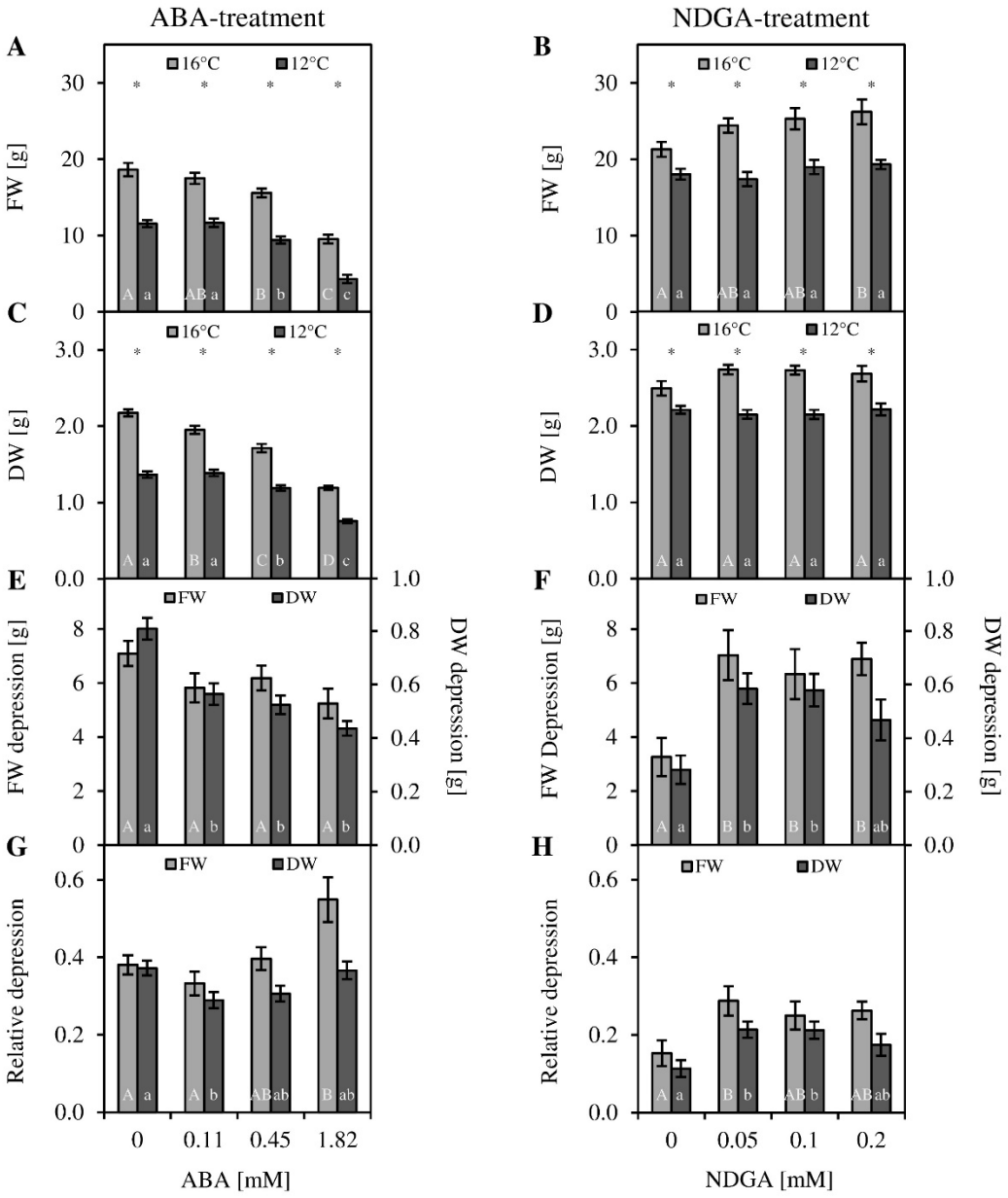
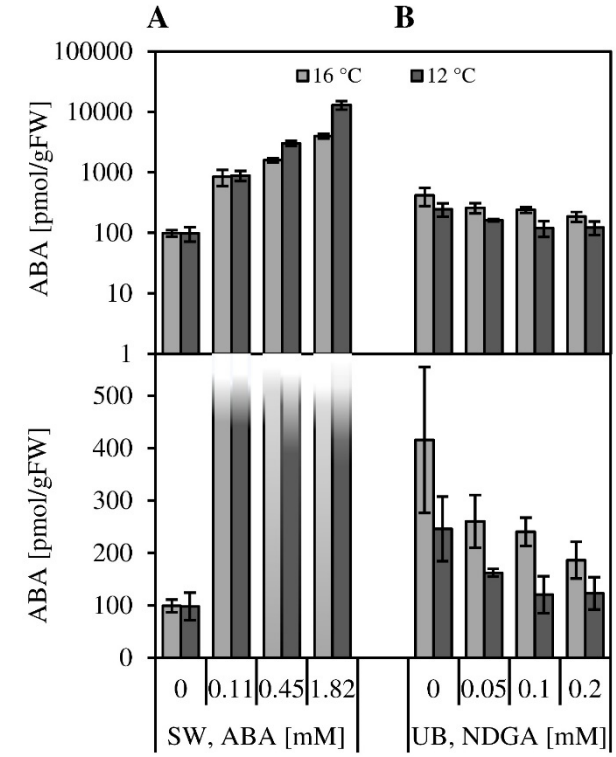


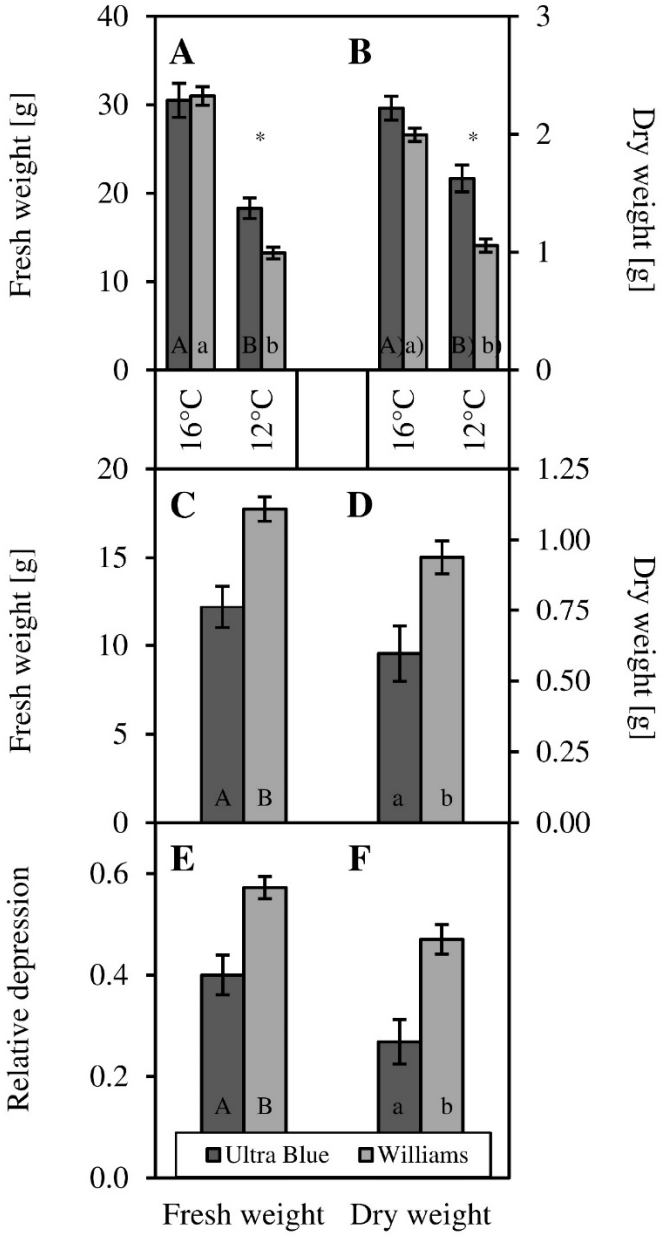
Fig. 12 Impact of pharmacological treatments on dry and fresh weight production. Impact of chilling on dry (DW) and fresh weight (FW) production during ABA-treatment on SW and NDGA-treatment on UB (A-D: light grey: 16 °C, dark grey: 12 °C; E, F: light grey: FW depression, dark grey: DW depression; G, H: light grey: FW ratio, dark grey: DW ratio). Shown are weight increases after three weeks. (A) FW of ABA-treated SW; (B) FW of NDGA-treated UB; (C) DW of ABA-treated SW; (D) DW of NDGA-treated UB; (E) FW and DW depression (16 °C - 12 °C) of ABA-treated SW; (F) FW and DW depression (16 °C - 12 °C) of NDGA-treated UB; (G) relative growth depression of FW and DW ((16 °C - 12 °C) / 16 °C) of ABA-treated SW; (H) relative growth depression of FW and DW ((16 °C - 12 °C) / 16 °C) of NDGA-treated UB. (n = 16; data are means ± SE; different characters indicate significant differences between treatments, * $P < 0.05$; asterisks indicate significant differences between control and chilling temperature for a specific pharmacological treatment, $P \leq 0.05$).



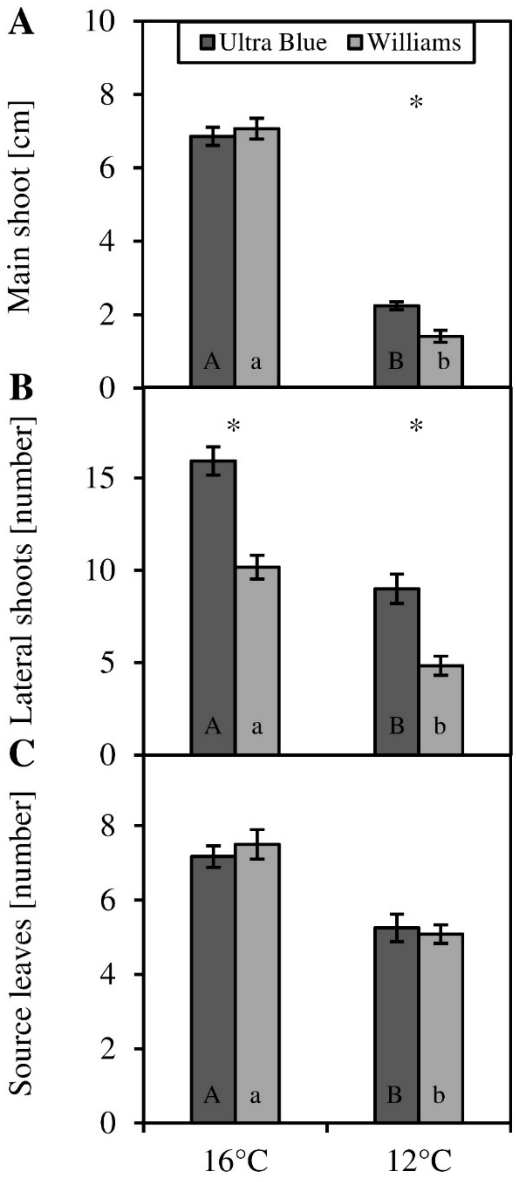
		ABA treatment	NDGA treatment
ABA	T	***	*
	Treat	***	*
	T x Treat	***	n.s.

Fig. 13 Effect of applications of ABA to SW and of NDGA to UB on ABA concentrations in the apex. ABA levels in apices of ABA treated SW and NDGA treated UB after exposure to control or chilling temperatures (light grey: 16 °C, dark grey: 12 °C). Samples were taken at 26 DoT, 7 days after the last spray application. (A) ABA concentrations of ABA-treated SW; (B) ABA concentrations of NDGA-treated UB. (n = 4; data are means ± SE; In the Significance Table, asterisks indicate significance levels of the effects of treatment (Treat) and temperature (T) and of interactions between Treat and T for the specified DoT, * $P \leq 0.05$, *** $P \leq 0.0001$; n.s. = not significant).

Additional files



Additional file 1: Figure S1. Dry weight, fresh weight, and growth depression. Impact of chilling on fresh and dry weight production. Shown are weight increases of aerial shoots after three weeks of exposure to chilling (grey: UB; white: SW). (A) fresh weight; (B) dry weight; (C) absolute growth depression of fresh weight; (D) absolute growth depression of dry weight; (E) relative growth depression of fresh weight (1 - 12 °C/16 °C); (D) relative growth depression of dry weight (1 - 12 °C/16 °C). (n = 12; data are means ± SE; asterisks indicate significant differences between cvs, * $P \leq 0.05$; different characters indicate significant temperature effects within one cv, $P < 0.05$).



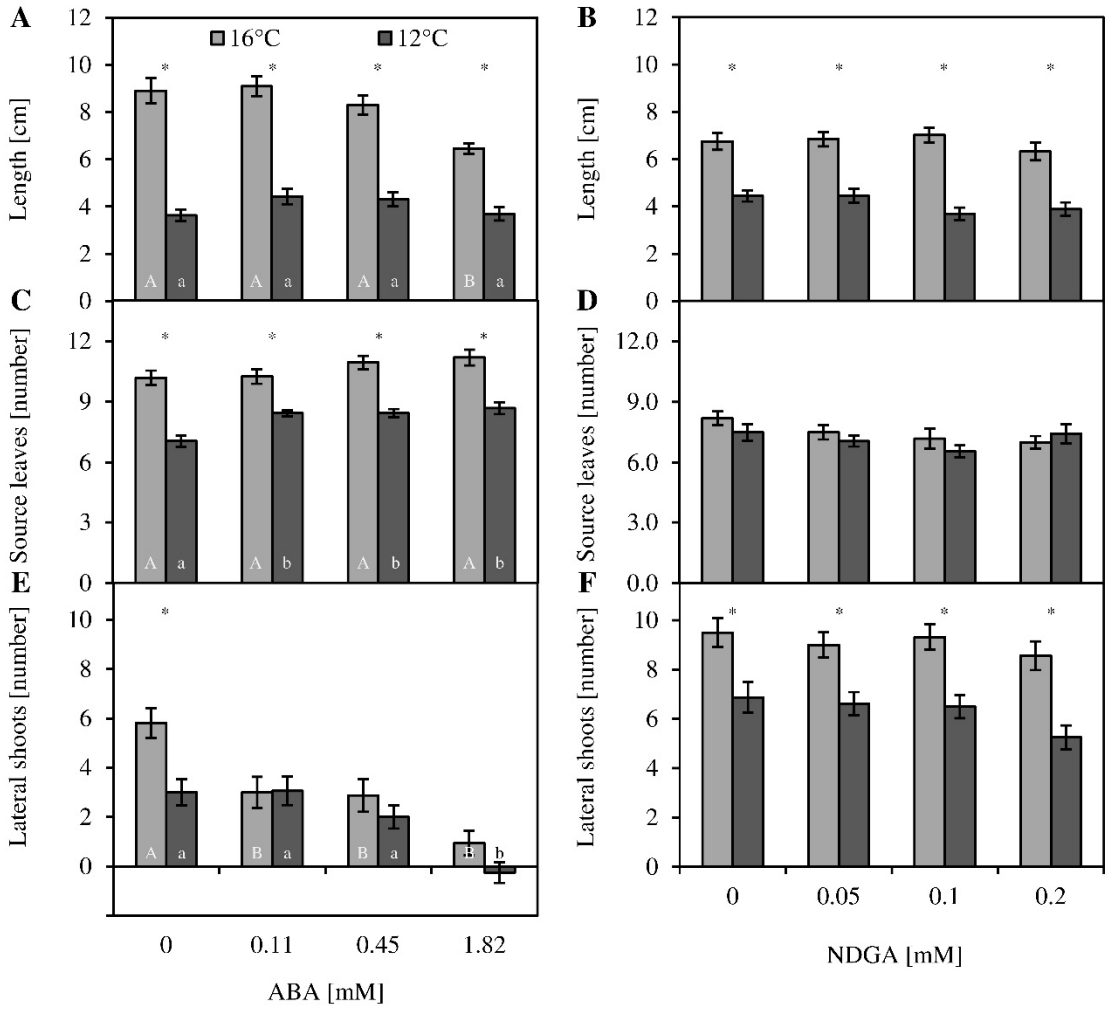
Additional file 2: Figure S2. Growth parameters. Impact of chilling on the length of main shoot and numbers of lateral shoots and leaves. Shown are increases after three weeks of exposure to the different temperatures (blue: UB; yellow: SW). (A) length increase of the main shoot; (B) number of newly developed lateral shoots; (C) number of newly developed leaves on the main shoot. (n = 12; data are means ± SE; asterisks indicate significant differences between cvs, * $P < 0.05$; different characters indicate significant temperature effects within one cv, $P < 0.05$).

Due to the large amount of data, it was not possible to print the additional files 3-5 here. Please find the regarding files on the enclosed CD-ROM (file names: ***Chapter_2.3_Tab_S1A.xlsx, Chapter_2.3_Tab_S1B.xlsx, Chapter_2.3_Tab_S1C.xlsx****):*

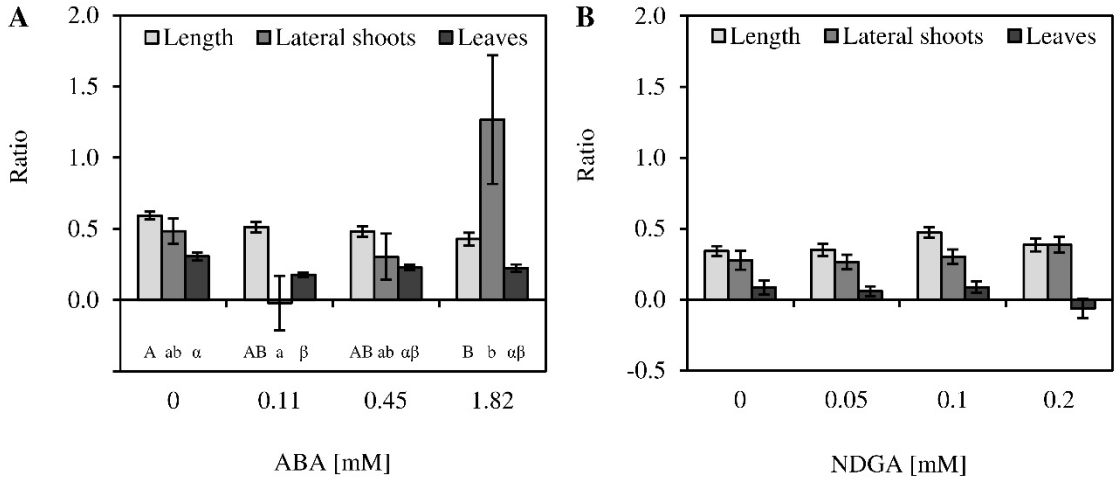
Additional file 3: Table S1A. Expression values, Ultra Blue. Means of normalized expression values of all analyzed petunia genes for each date.

Additional file 4: Table S1B. Expression values, Sweet Sunshine Williams. Means of normalized expression values of all analyzed petunia genes for each date.

Additional file 5: Table S1C. M-values of significant hybridization differences between UB and SW. M-values (Log2 of hybridization ratios between UB and SW) of genes which were at least at one date significantly differentially hybridized. Genes being more than 2-times higher or lower hybridized in UB compared to SW are highlighted in red or in green, respectively (M-value > 1 or < 1). Pfp-values < 0.15 identify significantly differentially hybridized genes and are highlighted in yellow. "Cond 1 < Cond 2 pfp value" are relevant for genes with lower hybridization rates in UB; "Cond 1 > Cond 2 pfp value" are relevant for genes with higher higher hybridization rates in UB.



Additional file 6: Figure S3. Impact of pharmacological treatments on growth parameters. Effect of treatment of the cv SW with abscisic acid (ABA) and of the cv UB with nordihydroguaiaretic acid (NDGA) on the length of main shoot and the numbers of lateral shoots and leaves. Shown are increases after three weeks of exposure to the different temperatures and treatments (light grey: 16 °C, dark grey: 12 °C). (A) length increase of the main shoot of ABA-treated SW; (B) length increase of the main shoot of NDGA-treated UB; (C) numbers of newly developed lateral shoots of ABA-treated SW; (D) numbers of newly developed lateral shoots of NDGA-treated UB; (E) numbers of newly developed leaves on the main shoot of ABA-treated SW; (F) numbers of newly developed leaves on the main shoot of NDGA-treated UB. (n = 16; data are means ± SE; different characters indicate significant differences between treatments, $P < 0.05$; asterisks indicate significant differences between control and chilling temperature for a specific pharmacological treatment, $P \leq 0.05$).



Additional file 7: Figure S4. Impact of pharmacological treatments on chilling induced growth depression in relation to cultivation at 16 °C. Effect of treatment of the cv SW with abscisic acid (ABA) and of the cv UB with nordihydroguaiaretic acid (NDGA) on the depressions of elongation of the main shoot (light grey), and of numbers of lateral shoots (dark grey) and leaves on the main shoot (black) produced during three weeks of chilling. Shown is the relative depression compared to the control ((16°C - 12 °C) / 16 °C). (A) ABA-treated SW; (B) NDGA-treated UB. (n = 16; data are means ± SE; different characters indicate different significance groups, $P \leq 0.05$).

3 Conclusions and outlook

For the production of plants, especially for thermophilic ornamentals like petunia, heating of greenhouses is the most important expense factor. Therefore, a production at reduced temperatures would save a huge amount of energy as well as emissions of greenhouse gases. However, this aim can only be achieved with cultivars, the growth of which is just slightly reduced by chilling and thus the production time is not much prolonged. The present thesis investigated how such cultivars can be identified and aimed at finding molecular patterns that contribute to a better growth performance under chilling. The results of this thesis are discussed in detail in the corresponding publications and manuscripts. The following chapters give a comprehensive overview of the results of this thesis and discuss the gained novel information, which can serve as a basis for future research.

3.1 Establishment of a screening system for chilling tolerance in *Petunia hybrida* and screening of cultivars with distinct chilling responses

The impact of chilling on commercially available *Petunia hybrida* cultivars varies depending on their genotype and the resulting growth habit. To identify cultivars differing in susceptibility to chilling, a set of *Petunia hybrida* cultivars described by growers as putatively chilling-sensitive or -tolerant, was evaluated for their phenotypical responses to chilling. Therefore, a chilling temperature was needed fulfilling two preconditions. First, it had to be reduced far enough below commonly used cultivation temperatures (= control) to trigger a mild chilling stress in the plants which is reflected by significant growth responses compared to the control. Secondly, the chilling temperature had to be high enough to prevent the plants from developing visible chilling injuries and to allow a growth performance that does not lead to exceptionally exceeding the normal production time. As average control temperature 16 °C was chosen, a temperature commonly used for German petunia production, and as average chilling temperature 12 °C was chosen. The latter was proven to accomplish both above-mentioned preconditions. The effect of this chilling temperature on the evaluated cultivars was even strong enough to find significant differences in the phenotypical chilling response already after three to four weeks, with a significantly weaker impact on growth in the putative chilling-tolerant cultivars (Chapter 2.1).

The analyzed growth parameters dry and fresh weight production, elongation of the main shoot, development of new leaves, and development of lateral shoots were reduced in response to chilling in most of the cultivars with exception of the production of lateral shoots (Chapter 2.1). Hereby, the chilling response of dry weight production was proven to be the most reliable and reproducible parameter. Even if the chilling-induced growth reduction was strongly determined by the general growth performance of the cultivars, distinct chilling responses could be identified between the

cultivars. While the absolute growth depression in terms of dry weight was highly variable between individual experiments, relative decreases in dry weight production under chilling could be reproduced at least for some cultivars that could be classified as chilling-tolerant or -sensitive, respectively.

While the development of lateral shoots was only marginally affected by chilling in most experiments, but featured high cultivar-specific temperature-independent differences, the data suggest that high-branching cultivars may be less susceptible to chilling. Thus, the number of newly developed lateral shoots was positively correlated at both temperatures with several relative growth parameters. Accordingly, one explanation for that finding might be that a higher branching rate indicates a higher number of growing points, that provide large utilization sinks for carbohydrates being produced in the source leaves (Lieth et al., 1991). Elongation growth and leaf development on the main shoot were reduced by chilling in tolerant cultivars as well as in sensitive ones. In contrast, the data suggest that a high-branching rate may contribute more to biomass production than a low-branching rate, since lateral shoots, as soon as leaves are big enough, are able to semi-autonomously supply themselves with carbon. Thus, higher branching might be a practical first selection criterion for screening of huge pools of *Petunia hybrida* cultivars for chilling-tolerant ones.

3.2 Introduction of growth indices for the evaluation of growth performance

The insights from this thesis improve the knowledge of how to select cultivars tolerant to mild sub-optimal temperatures from a given set of different petunia cultivars by recommending easily applicable growth indices, which can be very decisive for future breeding of chilling-tolerant cultivars. These could contribute to a more sustainable, energy and greenhouse gas saving production of petunia in moderate climates.

For the evaluation of the growth response to chilling in comparative approaches, specific indices were introduced (Chapter 2.1). The GI (growth index) and CPI (chilling performance index) describe the growth of an individual cultivar at control temperature or chilling, respectively, in relation to the best growing cultivar of the evaluated set at the same temperature. The best growing cultivar instead of the mean of all cultivars was chosen for this comparison to evaluate the relative growth potential of individual cultivars in relation to the maximum growth, defined by the best growing cultivar. The CTI (chilling tolerance index) describes the dry weight production under chilling in relation to control. This index can be used as a stand-alone index to evaluate the individual chilling response.

Since the above-mentioned indices (GI, CPI) compare the responses of the cultivars within an analyzed set, they rather estimate the relative chilling-tolerance potential in relation to the other analyzed cultivars than give absolute information of the chilling susceptibility of an individual

cultivar. The investigated cultivars displayed strong general, chilling-independent growth differences, especially in terms of dry/fresh weight production and development of lateral shoots. However, the relative growth performance under chilling vs. control conditions (CTI) revealed significant differences among the cultivars, which could be reproduced for a selection of five distinct chilling-susceptible cultivars in four experiments. Nevertheless, to verify if the introduced indices are also applicable on a large scale, they should be tested for screenings of higher numbers of cultivars. After successful verification, these indices could be used for the easy testing of bigger sets of cultivars to identify chilling-tolerant ones.

However, to validate the findings described in Chapter 3.1 and 3.2, additional screenings with larger sets of cultivars should be conducted. Additionally, screenings of other petunia species or chilling-tolerant wildtypes could be added to evaluate if these findings are also true and generalizable for petunia on the species level. Screenings of sets of *Petunia ssp*. with higher genetic diversity could also reveal further phenotypical markers in addition to the branching rate, which would be helpful in identifying chilling-tolerant genotypes. The presented investigations focused on the vegetative chilling response only in order to elucidate the basic growth response patterns of chilling tolerance in petunia. Nevertheless, for breeders and producers, especially the quality of the flowering plants are of huge interest. Therefore, future screenings should also include the evaluation of markers that estimate the flowering of the plants. Thus, it would be crucial to find out if chilling tolerance of the vegetative growth also correlates with a lower number of days to flower and/or higher numbers of flower buds. Since a minimum number of nodes below the first flower seems to be essential for flowering (Warner and Walworth, 2010), it is very likely that high CTIs – especially when combined with high leaf development rates - will correlate with only small delays in time to flower compared to normal production temperatures.

3.3 Comparative analysis of the chilling-tolerant cultivar ‘Ultra Blue’ and the chilling-sensitive cultivar ‘SweetSunshine Williams’

The cultivars ‘Ultra Blue’ and ‘SweetSunshine Williams’ were identified to serve as chilling-sensitive and -tolerant model cultivars and were chosen for the subsequent physiological and molecular investigations. Both cultivars reacted to the exposure to chilling with a clear growth depression, which was considerably stronger in ‘SweetSunshine Williams’. As already demonstrated in the initial screenings (Chapter 2.1), this growth retardation was mainly connected with a reduced elongation of the main shoot and a reduction in the production of new leaves, but for the production of new lateral shoots, genotypic differences were more pronounced. The present results suppose that the detected growth depressions are the consequence of a functional disturbance of the whole plant, caused by a highly dynamic and complex molecular physiological stress response, which was more severe in the

chilling-sensitive cultivar. This was featured by changes in phytohormone homeostasis, carbohydrate metabolism, and gene expression patterns in source leaves, apex and the stem, which reflect important functional units for plant growth. Nevertheless, differences in tolerance to mild chilling temperatures between 'SweetSunshine Williams' and 'Ultra Blue' seem not only to be based on different responses under chilling, but also on general differences in carbohydrate metabolism, phytohormonal regulation and gene expression between the two genotypes (Chapter 2.3).

The three proposed phases of chilling response, described in the characterization of 'SweetSunshine Williams' (Chapter 2.2) were not completely reproduced during the comparison of both cultivars (Chapter 2.3). There are different reasons that could explain this finding. First, the petunia plant is a very complex system that sensibly responds to environmental influences. Even when maintaining all environmental conditions stable, uncontrollable factors like the season of the year when cuttings are harvested from the mother plants might influence the conditioning and particularly the dynamic of metabolic reactions of the plants. Exemplary, despite short day cultivation of mother plants all over the year, flower induction on cuttings was increased and simultaneously rooting rates were reduced during the summer season. Further, for phytohormone measurements and gene expression analyses, the first definable internode was used for the characterization of 'SweetSunshine Williams'. However, after three to four weeks of the experiment, the size and position in the plant architecture of this first collectable internode varied distinctly between chilled and control plants. Thus, for a more consistent sampling in the comparison of cultivars, a defined shoot section, without leaves, situated directly below the sampled apex, was collected. Consequently, differences in internodium and upper shoot results might be based on the different sampling and cannot be used for the evaluation of reproducibility. Nevertheless, the general strong disturbance of carbohydrate metabolism in Williams at the source side and the obvious decoupling of leaf carbohydrates from the growth sink in response to chilling was reproduced. Finally, the characterization of gene expression of 'SweetSunshine Williams' by array analysis focused on the differences under chilling in comparison to control to feature the chilling response. In contrast, the comparison of the two cultivars compared hybridization rates of genes between these cultivars at both temperatures separately to find distinct chilling responses but also general cultivar-specific differences by simultaneously considering transcript levels of the genes. However, since general metabolic differences as well as the general direction of chilling response seem to be more crucial for chilling tolerance than transient response phases, the differences found in the comparative analysis are predominantly discussed below.

3.4 Chilling tolerance correlates with a more stable leave carbohydrate metabolism and a better supply of and utilization in the apex

The carbohydrate metabolism is essential for plant growth. Equally important are the availability of carbon sources in the plant, the transport from source tissues to growing sinks, as well as the utilization at the site of growing sinks. In the present thesis, chilling caused a derangement of the homeostasis of soluble sugars in both cultivars, whereas the leaf carbohydrate metabolism of the chilling-tolerant cultivar 'Ultra Blue' was more stable and its shoot apex showed a generally higher abundance and utilisation of sucrose (Chapter 2.3). In contrast to cold and freezing temperatures, only few studies have investigated the chilling response of carbohydrate metabolism at mild sub-optimal temperatures. However, while it is widely known that soluble sugars increase in plant cells at very low temperatures to shelter cells as cryoprotectants from freezing (reviewed in Mahajan and Tuteja, 2005), such increases are already reported for moderate sub-optimal temperatures. Thus, Venema et al. (1999) found increased carbohydrate concentrations and starch accumulation in tomatoes' youngest mature source leaves as a response to mild chilling (day/night temperature, control: 20/25 °C; chilling: 16/14 °C). The authors observed that the reduction of biomass production, particularly of new leaves, and in one genotype also the leaf expansion, could be correlated to sub-optimal temperature, even without changes in photosynthetic rates. Thus, they related the strong accumulation of carbohydrates, especially in the form of starch, which they observed in sub-optimally developed tomato leaves, to the magnitude of chilling stress (Venema et al., 1999). These findings comply with the stronger increases in hexose and sucrose concentrations in the chilling-sensitive cultivar presented in this thesis, even if the differences in starch accumulation under chilling between both cultivars were only marginally. However, the stronger starch accumulation in the apex of the chilling-tolerant cultivar, especially in combination with the higher sucrose levels, and cytINV and cwINV activities in the apex, might indicate a general better carbohydrate translocation and utilization in the growing apical tissue of 'Ultra Blue'. The constitutively higher sucrose levels in the apex of 'Ultra Blue' did not seem to be the result of altered expressions of genes coding for sucrose phosphate synthase or sucrose synthase in comparison to 'SweetSunshine Williams'. Thus, the different levels may rather be the result of post-transcriptional regulation of sucrose synthesis and/or degradation and translocation, respectively, or these enzymes are not cultivar-specifically regulated at all. However, there is also a possibility that genes coding for the responsible enzymes are not covered by the microarray. Moreover, higher invertase activities and lower expression rates of genes coding for invertase inhibitors indicate improved translocation and utilization of sucrose via action of invertases. Additionally, since photosynthetic rates were generally lower in 'SweetSunshine Williams' compared to 'Ultra Blue', and did not change under chilling in both cultivars, the higher increases in sugar levels under chilling in the source leaves of 'SweetSunshine Williams' cannot be explained by higher photosynthetic rates. Nevertheless, since the gene expression analysis did not reveal an involvement

of putative genes controlling photosynthesis, other causes might be responsible for the cultivar-differences in photosynthetic rates, like stomata regulation and density or post-transcriptional regulation of essential proteins. Nevertheless, it cannot be excluded that genes are affected whose function is not annotated yet, or which are not present on the microarray. Chapter 2.3, discusses in detail several genes whose hybridization rates suggest a better carbon supply of source leaves by open stomata, an improved translocation of photosynthetic products from chloroplasts to the cytosol of source leaves, as well as a better carbohydrate translocation from source to sink tissues for the chilling-tolerant cultivar 'Ultra Blue'. Further, transcriptome data suggest that an improved channeling of carbon flux from glycolysis towards cataplerotic reactions via the citric cycle and thus to biosynthesis in the apex seems to be driven by higher phosphoenolpyruvate carboxylase (PEP carboxylase) kinase levels and an enhanced citric cycle flux. This higher affinity towards cataplerotic pathways combined with the higher osmotic potential in the apical tissue, caused by the higher Suc abundance, which is further increased by enhanced invertase activities, might increase cell elongation and plant growth at the growing points of 'Ultra Blue' (Gibeaut et al., 1990).

Finally, the findings of a less disturbed carbohydrate metabolism of the source leaves and the higher sucrose abundance and utilization in the apex of 'Ultra Blue' suppose that the chilling response of 'Ultra Blue' is shifted more to a growth priority strategy. In contrast, the chilling response of carbohydrate metabolism in 'SweetSunshine Williams' is more shifted towards a primary defense strategy to protect the important maintenance function of the source leaves already at this mild chilling. To test the hypothesis, whether increased activities of cytINV and cwINV contribute to a higher carbon abundance and utilization in the apex, invertase activities in the apex of 'SweetSunshine Williams' could be manipulated by the use of apical-meristem-specific promoters for the overexpression of invertase genes. Additionally, the efficiency of invertases could be enhanced by RNAi-mediated degradation of transcripts coding for invertase inhibitors. In the same way, the significance of PEP carboxylase for a growth priority strategy could be tested by overexpression of genes for PEP carboxylase and PEP carboxylase kinase.

3.5 Higher apical ABA levels are correlated with chilling tolerance

Phytohormones are essential for numerous reactions in plants. They play key roles in plant development, growth and the response to environmental stimuli. The observed fundamental differences in phytohormone levels, particular in the apex, and the related array results support a higher growth priority of 'Ultra Blue'. Thus, the results suggest especially for ABA a key role for chilling tolerance (Chapter 2.3). Besides, IAA seems to have only a minor role and JA seems to be not involved at all in chilling tolerance of 'Ultra Blue' at this mild stage of chilling. Particularly in the apex of the chilling-tolerant cultivar constitutively higher ABA levels were found. It has to be

considered, that depending on the concentration, ABA has not only a growth retarding function, but can also improve stress resistance (Sreenivasulu et al., 2012). Thus, whereas Mega et al. (2015) described that low ABA levels contributed to seedlings vigor in rice under cold stress, several other studies proved an important role of ABA for the tolerance to chilling and cold stress (Veisz et al., 1996; Ntatsi et al., 2013; Yang et al., 2015). In the present thesis, the constitutive higher ABA levels especially in the apex might have supported the higher chilling tolerance as reflected by the enhanced dry matter production and relative growth under chilling compared to control in comparison to 'SweetSunshine Williams'. The higher ABA levels also correlated with constitutively higher hybridization rates of a gene coding for a carotenoid cleavage dioxygenase, similar to the 9-cis-epoxycarotenoid dioxygenase, which catalyzes xanthophyll cleavage, the first and rate-limiting step of ABA biosynthesis (Saito et al., 2006). Since ABA signaling is known to influence carbohydrate metabolism and regulate invertases (Trouverie et al., 2003, 2004; Ren et al., 2015), the findings of the higher ABA levels in 'Ultra Blue' apices also correlate with the higher sucrose levels and higher invertase activities. Additionally, the array data also suggest lower hybridization rates of genes coding for enzymes of the ethylene synthesis pathway, which might also be correlated with the higher ABA levels. Normal ABA levels of unstressed plants are vital for maintaining shoot development and leaf expansion, which is partly realized by a restriction of ethylene biosynthesis and/or sensitivity (LeNoble et al., 2004). The maintenance of ABA levels and simultaneous reduction of ethylene biosynthesis might be essential to sustain the growth performance under chilling also for further reasons. Thus, the CBF-induced cold tolerance pathway is reported to be negatively regulated by ethylene signaling, whereas a chilling-induced decrease in ethylene biosynthesis seems to be important for an activation of this pathway (Shi et al., 2012). However, in the present work no indications for an activation of the CBF-pathway were found.

Pharmacological approaches can be used to manipulate phytohormone levels to elucidate hormone functions in regulation of growth in general and under stress in particular. Therefore, considering the above-mentioned results, a pharmacological treatment was applied for exogenous manipulation of ABA levels. Thus, to enhance the ABA levels in the chilling sensitive cultivar, ABA was applied, and to reduce the ABA levels in the chilling-tolerant cultivar the ABA synthesis inhibitor NDGA was applied. Nevertheless, to achieve an internal phytohormone distribution within the plant that mimics the homeostasis regulated by the plant itself in dependence on its genetic background and in response to the environment is principally challenging. This is especially true in the case of ABA, which can enhance stress tolerance or even retard growth just depending on its concentration (Sreenivasulu et al., 2012). However, the ABA treatment enhanced and the NDGA-treatment reduced the ABA levels in the apex of the treated plants. The growth responses of the treated plants to chilling suppose a role of ABA in chilling tolerance. Nevertheless, the tolerance promoting effect seems to be highly dependent on the intrinsic distribution of ABA within the plant. The results support the hypothesis that higher

ABA levels enhance the effectiveness of the stress response machinery, especially at the growing meristems of the plant, which are crucial for biosynthesis of new plant material.

In conclusion, the protective function of ABA under chilling seems to be connected not only to the overall ABA level of a plant but in particular to be dependent on a fine-tuned ABA homeostasis between particular plant organs, when comparing the distribution of ABA between the different plant tissues in 'Ultra Blue' and 'SweetSunshine Williams'. To explore a better knowledge of these regulations further research is necessary by applying modified expression of genes coding for enzymes of the ABA biosynthesis pathways under the control of tissue-specific promotors. The above-mentioned constitutively higher hybridization levels of a gene coding for carotenoid cleavage dioxygenase in 'Ultra Blue' might indicate a cultivar-specific up-regulation of this gene. But more likely, these general higher hybridization rates are a result of a polymorphism of this gene. This would allow the possibility that the resulting enzyme might display differences in activity between both cultivars. To evaluate, if carotenoid cleavage dioxygenase plays a role for chilling tolerance at all, an apex-specific constitutive overexpression could be applied to up-regulate ABA levels locally in the apex of 'SweetSunshine Williams'. In the case of ABA, a tissue-specific expression would be of special interest to simultaneously avoid the growth-retarding effects of ABA, when higher expressed in the source leaves (Estrada-Melo et al., 2015). Since excessive ABA degradation might reduce cold tolerance (Mega et al., 2015), the degradation pathway would also be an interesting target for manipulating ABA levels in the plant. The most effective way to block the degradation of ABA seems to be the inhibition of the cytochrome P450 enzyme ABA 8'-hydroxylase (Cutler and Krochko, 1999). Such an inhibition of ABA degradation would result in an ABA accumulation.

3.5.1 Other candidate processes of chilling tolerance supported by differences in gene expression

For the characterization of the chilling-sensitive cultivar 'SweetSunshine Williams', the gene expression analysis focused on differential expression of genes under chilling compared to the control temperature (Chapter 2.2). Applying this scope of analysis on the comparison of both cultivars, only a few genes were found to be up- or down-regulated simultaneously in both cultivars, whereas most chilling-responsive genes were differentially regulated only in one of both cultivars (Chapter 2.3). The data suppose differences in gene expression between both cultivars, especially in the source tissue during the early phase of chilling, which is essential for the maintenance of carbohydrate production and energy supply. Since this approach only focused on relative differences between the temperature treatments, but did not consider absolute transcript levels of the analyzed sequences, in a second approach the hybridization rates of both cultivars were compared directly, and separately for each temperature. Genes differentially hybridized only under chilling, featured organ specific patterns and

different consecutive phases in chilling response. Whereas the differences in the apex attenuated with the duration of chilling treatment, differences in the upper stem increased again after a transient weakening in differences after one week. In the source tissue merely the quality of involved functional groups changed from a higher number of higher expressed genes in 'Ultra Blue' in the early phase to a higher number of lower expressed genes in 'Ultra Blue' in the late phase. These higher expression rates in the early phase might suggest a faster chilling response of more chilling-regulated genes in 'Ultra Blue'. However, also many genes were found to be constitutively differentially hybridized at both temperatures, some of them in all tissues and at all dates. Latter might rather be the result of polymorphisms than of distinct expressions.

Under the applied mild chilling stress, the CBF-pathway, heat-shock proteins and cold-induced proteins did not seem to play a significantly different role between both cultivars. Thus, they most likely also did not have a dominant role for chilling tolerance of 'Ultra Blue'. Furthermore, the chilling-responsive transcription factors that were revealed by the characterization of 'SweetSunshine Williams' did not display clear cultivar-specific response patterns, when hybridization rates of genes were compared between both cultivars. The Jumonji (JmjC) domain-containing protein was one of the most constitutively chilling-dependent up-regulated genes in the characterization of the chilling-sensitive cultivar 'SweetSunshine Williams'. However, since it was up-regulated at similar transcript levels in both cultivars, it was not mentioned in the comparison. Although these results suggest a role of this gene in the chilling response, it did not seem to be essential for the distinct reactions of the two cultivars analyzed.

The results of this thesis generate a better understanding of the molecular mechanisms of chilling tolerance, and suggest candidate genes that might be involved in chilling tolerance. The better understanding how to determine chilling tolerance and the insights of the importance of a stable carbohydrate-translocation to the utilization sinks as well as the protective function of ABA in the apex can be very helpful for the future selection and breeding of more chilling-tolerant cultivars. However, further investigations are indispensable to elucidate if the above-discussed models of two distinct chilling-susceptible cultivars can be generalized to explain the chilling responses of other petunia cultivars too. Thus, carbohydrate metabolism, phytohormone levels and gene expression of other cultivars should be evaluated. Furthermore, the candidate genes controlling the discussed metabolic and plant hormone pathways should be further tested. This can be executed by the use of knock-out mutants, by knock-downs using RNAi technology or the overexpression of genes coding for enzymes like 9-cis-epoxycarotenoid dioxygenase, phosphoenolpyruvate carboxylase kinase or invertases and invertase inhibitors with involvement of tissue-specific promotors.

4 Confirmation of the three thesis objectives

The three main goals of this thesis were described in Chapter **1.4**. In the following, the objectives are mentioned again and a short overview, to which extend they were achieved, is given.

1.) Establishment of a reliable and easily applicable screening method for the identification of cultivars with chilling-tolerant and chilling-sensitive responses, respectively.

A temperature reduction of 4 Kelvin from commonly used production temperatures for petunia (16 °C to 12 °C with 2 K day/night-difference each) was proven to be an appropriate testing system to reveal the distinct susceptibilities to chilling in a set of cultivars. Dry weight production was shown to be the most reliable and reproducible growth parameter for evaluating the level of chilling tolerance. Growth indices of dry weight production of an individual cultivar in a set of cultivars were established to assess the relative growth performance. These indices were calculated in relation to the best performing cultivar of the analyzed set at a given temperature to estimate the growth potential of a cultivar under the given conditions. Growth indices calculated for an individual cultivar at chilling and control temperatures gave information of the individual level of susceptibility to chilling stress.

2.) Characterization of the chilling reaction of a chilling-sensitive cultivar on the levels of phenotypical development, carbohydrate metabolism, phytohormone homeostasis and gene expression.

In several experiments, the cultivar 'SweetSunshine Williams' constantly showed a more severe susceptibility to chilling measured by relative decreases in dry weight production compared to the set of investigated cultivars. The characterization of the plants' response to chilling revealed a complex disturbance of plant functional integrity, visible at the levels of carbohydrates, phytohormones, and gene expression. Based on all findings, a response model with three consecutive phases was proposed for this chilling-sensitive cultivar. In the beginning, chilling led to a phase of destabilization of metabolic parameters and gene expression. This phase was followed by a transient recovery phase, marked by a normalization of metabolic parameters and a lower number of differentially expressed genes. This phase was followed by a phase of stabilization, indicating a long-term acclimation to chilling. These adaptions were suggested to help the plant to cope with the stress induced by chilling without damage but at the expense of growth.

3.) Formulation of a hypothesis for the basis of chilling tolerance in *P. hybrida*, which is based on the distinct reaction patterns of the phenotypical development, the carbohydrate metabolism, the phytohormone homeostasis and the gene expression of a chilling-sensitive and a chilling-tolerant cultivar and the identification of new candidate genes involved in chilling tolerance.

The chilling responses of the chilling-sensitive cultivar ‘SweetSunshine Williams’ and of the chilling-tolerant cultivar ‘Ultra Blue’ displayed obvious differences. However, also general cultivar-specific, chilling-independent differences between both cultivars seem to be important for the differences in chilling tolerance. Thus, metabolic parameters as well as gene expression data suppose that the tolerant cultivar has a more stable carbohydrate metabolism in the source tissue under chilling, but also a generally better carbohydrate supply and utilization in the apex tissue. Despite the growth-inhibiting role of high ABA levels, higher ABA concentrations in the apex may additionally support a better growth under chilling. ABA-treatment of the chilling-sensitive cultivar and ABA inhibitor (NDGA)-treatment of the chilling-tolerant cultivar seemed to confirm the role of ABA in chilling tolerance. Candidate genes for carbohydrate metabolism and translocation as well as for ABA synthesis such as invertase inhibitors and phosphoenolpyruvate carboxylase kinase or carotenoid cleavage dioxygenase were identified.

5 References

Abeles, F. B., Morgan, P. W., and Saltveit, M. E. (1992). "The role of ethylene in agriculture," in *Ethylene in Plant Biology* (Elsevier), 264–296. doi:10.1016/B978-0-08-091628-6.50015-1.

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J., and Harberd, N. P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. *Science* 311, 91–4. doi:10.1126/science.1118642.

Adams, S. R., Hadley, P., and Pearson, S. (1998). The effects of temperature, photoperiod, and photosynthetic photon flux on the time to flowering of petunia "Espress Blush Pink." *J. AMER. Soc. Hort. Sci.* 123, 577–580.

Adams, S. R., Pearson, S., Hadley, P., and Patefield, W. M. (1999). The effects of temperature and light integral on the phases of photoperiod sensitivity in *Petunia x hybrida*. *Ann. Bot.* 83, 263–269. doi:10.1006/anbo.1998.0817.

Ahkami, A. H., Lischewski, S., Haensch, K.-T., Porfirova, S., Hofmann, J., Rolletschek, H., Melzer, M., Franken, P., Hause, B., Druege, U., et al. (2009). Molecular physiology of adventitious root formation in *Petunia hybrida* cuttings: involvement of wound response and primary metabolism. *New Phytol.* 181, 613–625. doi:10.1111/j.1469-8137.2008.02704.x.

Ahkami, A., Scholz, U., Steuernagel, B., Strickert, M., Haensch, K.-T., Druege, U., Reinhardt, D., Nouri, E., von Wirén, N., Franken, P., et al. (2014). Comprehensive transcriptome analysis unravels the existence of crucial genes regulating primary metabolism during adventitious root formation in *Petunia hybrida*. *PLoS One* 9, e100997. doi:10.1371/journal.pone.0100997.

Allen, D. J., and Ort, D. R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends Plant Sci.* 6, 36–42. doi:10.1016/S1360-1385(00)01808-2.

Bagnall, D. J., King, R. W., and Farquhar, G. D. (1988). Temperature-dependent feedback inhibition of photosynthesis in peanut. *Planta* 175, 348–354. doi:10.1007/BF00396340.

Bauerfeind, M. A., Winkelmann, T., Franken, P., and Druege, U. (2015). Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress. *Front. Plant Sci.* 6:583. doi:10.3389/fpls.2015.00583.

Blanchard, M. G., Runkle, E. S., and Fisher, P. R. (2011a). Modeling plant morphology and development of petunia in response to temperature and photosynthetic daily light integral. *Sci. Hortic. (Amsterdam).* 129, 313–320. doi:10.1016/j.scienta.2011.03.044.

Blanchard, M. G., Runkle, E. S., and Frantz, J. M. (2011b). Energy-efficient greenhouse production of petunia and tagetes by manipulation of temperature and photosynthetic daily light integral. in *Acta Horticulturae*, 857–864.

BMEL (2014). Verbrauch von Blumen und Zierpflanzen. *Der Gartenbau Deutschl. - Daten und Fakten. Bundesministerium für Ernährung und Landwirtschaft*, 30 – 31. Available at: http://berichte.bmelv-statistik.de/GBB-0000100-2012.pdf. Accessed 05 Mar 2016.

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., Hause, B., Bucher, M., Kretzschmar, T., Bossolini, E., et al. (2010). Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. *Plant J.* 64, 1002–1017. doi:10.1111/j.1365-313X.2010.04385.x.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M., and Zhu, J.-K. (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* 17, 1043–54. doi:10.1101/gad.1077503.

Chinnusamy, V., Zhu, J., and Zhu, J.-K. (2007). Cold stress regulation of gene expression in plants.

Trends Plant Sci. 12, 444–451. doi:10.1016/j.tplants.2007.07.002.

Chinnusamy, V., Zhu, J.-K., and Sunkar, R. (2010). Gene regulation during cold stress acclimation in plants. *Methods Mol. Biol.* 639, 39–55. doi:10.1007/978-1-60761-702-0.

Cutler, A. J., and Krochko, J. E. (1999). Formation and breakdown of ABA. *Trends Plant Sci.* 4, 472–478. doi:10.1016/S1360-1385(99)01497-1.

Ding, C. K., Wang, C. Y., Gross, K. C., and Smith, D. L. (2002). Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. *Planta* 214, 895–901. doi:10.1007/s00425-001-0698-9.

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 Biol. Technol.* 47, 126–135. doi:10.1016/j.postharvbio.2007.06.008.

Du, H., Liu, H., and Xiong, L. (2013). Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Front. Plant Sci.* 4, 397. doi:10.3389/fpls.2013.00397.

Elings, A., Kempkes, F. L. K., Kaarsemaker, R. C., Ruijs, M. N. A., van de Braak, N. J., and Dueck, T. A. (2005). The energy balance and energy-saving measures in greenhouse tomato cultivation. *Acta Hortic.* 691, 67–74. doi:10.17660/ActaHortic.2005.691.5.

Estrada-Melo, A. C., Chao, M., Reid, M. S., and Jiang, C.-Z. (2015). Overexpression of an ABA biosynthesis gene using a stress-inducible promoter enhances drought resistance in petunia. *Hortic. Res.* 2, 15013. doi:10.1038/hortres.2015.13.

Fung, R. W. M., Wang, C. Y., Smith, D. L., Gross, K. C., and Tian, M. (2004). MeSA and MeJA increase steady-state transcript levels of alternative oxidase and resistance against chilling injury in sweet peppers (*Capsicum annuum* L.). *Plant Sci.* 166, 711–719. doi:10.1016/j.plantsci.2003.11.009.

Gabot.de (2012). GABOT : Gartenbau : Energiekrise bedroht Konkurrenzfähigkeit. Available at: http://www.gabot.de/index.php/News-Details/52/0/?&tx_ttnews[tt_news]=226333tx_ttnews[backPid]= 1&cHash=6cffc84d7d [Accessed May 7, 2016].

Gallie, D. R. (2015). Ethylene receptors in plants - why so much complexity? *F1000Prime Rep.* 7, 1–12. doi:10.12703/P7-39.

Garrido, G., Ramon Guerrero, J., Angel Cano, E., Acosta, M., and Sanchez-Bravo, J. (2002). Origin and basipetal transport of the IAA responsible for rooting of carnation cuttings. *Physiol. Plant.* 114, 303–312. doi:10.1034/j.1399-3054.2002.1140217.x.

Gerats, T., and Vandenbussche, M. (2005). A model system for comparative research: Petunia. *Trends Plant Sci.* 10, 251–256. doi:10.1016/j.tplants.2005.03.005.

Gibeaut, D. M., Karuppiah, N., Chang S-R, Brock, T. G., Vadlamudi, B., Kim, D., Ghosheh, N. S., Rayle, D. L., Carpita, N. C., and Kaufman, P. B. (1990). Cell wall and enzyme changes during the graviresponse of the leaf-sheath pulvinus of oat (*Avena sativa*). *Plant Physiol.* doi:10.1104/pp.94.2.411.

Gilmour, S. J., Zarka, D. G., Stockinger, E. J., Salazar, M. P., Houghton, J. M., and Thomashow, M. F. (1998). Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J.* 16, 433–442. doi:10.1046/j.1365-313X.1998.00310.x.

Goldschmidt, E. E., and Huber, S. C. (1992). Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiol.* 99, 1443–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16669056.

Gusta, L. V., Trischuk, R., and Weiser, C. J. (2005). Plant cold acclimation: The role of abscisic acid. *J. Plant Growth Regul.* 24, 308–318. doi:10.1007/s00344-005-0079-x.

Guy, C. L., Huber, J. L., and Huber, S. C. (1992). Sucrose phosphate synthase and sucrose accumulation at low temperature. *Plant Physiol.* 100, 502–508. doi:10.1104/pp.100.1.502.

Hällgren, J.-E., and Öquist, G. (1990). "Adaptions to low temperatures," in *Stress Responses in Plants: Adaption and Acclimation Mechanisms* (Wiley-Liss, Inc.), 265–293.

Hogewoning, S. W., and Harbinson, J. (2007). Insights on the development, kinetics, and variation of photoinhibition using chlorophyll fluorescence imaging of a chilled, variegated leaf. *J. Exp. Bot.* 58, 453–463. doi:10.1093/jxb/erl219.

Hu, Y., Jiang, L., Wang, F., and Yu, D. (2013). Jasmonate regulates the INDUCER OF CBF EXPRESSION-C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 cascade and freezing tolerance in *Arabidopsis*. *Plant Cell* 25, 2907–2924. doi:10.1105/tpc.113.112631.

Janská, A., Marsík, P., Zelenková, S., and Ovesná, J. (2010). Cold stress and acclimation - what is important for metabolic adjustment? *Plant Biol. (Stuttg).* 12, 395–405. doi:10.1111/j.1438-8677.2009.00299.x.

Kaczperski, M. P., Carlson, M. G., and Karlsson, M. G. (1991). Growth and development of *Petunia* × *hybrids* as a function of temperature and irradiance. *J. AMER. Soc. Hort. Sci.* 116, 232–237.

Kelly, R. O., Deng, Z., and Harbaugh, B. K. (2007). Evaluation of 125 petunia cultivars as bedding plants and establishment of class standards. *Horttechnology* 17, 386–396.

Knight, H., Zarka, D. G., Okamoto, H., Thomashow, M. F., and Knight, M. R. (2004). Abscisic acid induces *CBF* gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiol.* 135, 1710–1717. doi:10.1104/pp.104.043562.

Lee, B., Henderson, D. A., and Zhu, J.-K. (2005). The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell* 17, 3155–3175. doi:10.1105/tpc.105.035568.

LeNoble, M. E., Spollen, W. G., and Sharp, R. E. (2004). Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. *J. Exp. Bot.* 55, 237–45. doi:10.1093/jxb/erh031.

Li, B., Ning, L., Zhang, J., Bao, M., and Zhang, W. (2015). Transcriptional profiling of petunia seedlings reveals candidate regulators of the cold stress response. *Front. Plant Sci.* 6, 118. doi:10.3389/fpls.2015.00118.

Lieth, J. H., Merritt, R. H., and Kohl, H. C. (1991). Crop productivity of petunia in relation to photosynthetically active radiation and air temperature. *J. AMER. Soc. Hort. Sci.* 116, 623–626.

Mahajan, S., and Tuteja, N. (2005). Cold, salinity and drought stresses: An overview. *Arch. Biochem. Biophys.* 444, 139–158. doi:10.1016/j.abb.2005.10.018.

Mega, R., Meguro-Maoka, A., Endo, A., Shimosaka, E., Murayama, S., Nambara, E., Seo, M., Kanno, Y., Abrams, S. R., and Sato, Y. (2015). Sustained low abscisic acid levels increase seedling vigor under cold stress in rice (*Oryza sativa* L.). *Sci. Rep.* 5, 13819. doi:10.1038/srep13819.

Meyer, P., Linn, F., Heidmann, I., Meyer, H., Niedenhof, I., and Saedler, H. (1992). Endogenous and environmental factors influence 35S promoter methylation of a maize *A1* gene construct in transgenic petunia and its colour phenotype. *MGG Mol. Gen. Genet.* 231, 345–352. doi:10.1007/BF00292701.

Nagarajan, S., and Nagarajan, S. (2010). "Abiotic tolerance and crop improvement," in *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation* (Dordrecht: Springer Netherlands), 1–11. doi:10.1007/978-90-481-3112-9.

Ntatsi, G., Savvas, D., Druege, U., and Schwarz, D. (2013). Contribution of phytohormones in

alleviating the impact of sub-optimal temperature stress on grafted tomato. *Sci. Hortic. (Amsterdam).* 149, 28–38. doi:10.1016/j.scienta.2012.09.002.

Peleg, Z., and Blumwald, E. (2011). Hormone balance and abiotic stress tolerance in crop plants. *Curr. Opin. Plant Biol.* 14, 290–295. doi:10.1016/j.pbi.2011.02.001.

Raghavendra, A. S., Gonugunta, V. K., Christmann, A., and Grill, E. (2010). ABA perception and signalling. *Trends Plant Sci.* 15, 395–401. doi:10.1016/j.tplants.2010.04.006.

Rahman, A. (2013). Auxin: a regulator of cold stress response. *Physiol. Plant.* 147, 28–35. doi:10.1111/j.1399-3054.2012.01617.x.

Ren, X. D., Liu, H. M., Liu, Y. H., Hu, Y. F., Zhang, J. J., and Huang, Y. B. (2015). Influence of sugars and hormones on the genes involved in sucrose metabolism in maize endosperms. *Genet. Mol. Res.* 14, 1671–1678. doi:10.4238/2015.March.6.13.

Rietveld, P. L., Wilkinson, C., Franssen, H. M., Balk, P. A., van der Plas, L. H., Weisbeek, P. J., and Douwe de Boer, A. (2000). Low temperature sensing in tulip (*Tulipa gesneriana* L.) is mediated through an increased response to auxin. *J. Exp. Bot.* 51, 587–94. doi:10.1093/jexbot/51.344.587.

Roitsch, T. (1999). Source-sink regulation by sugar and stress. *Curr. Opin. Plant Biol.* 2, 198–206. doi:10.1016/S1369-5266(99)80036-3.

Ruelland, E., and Collin, S. (2016). "Chilling stress.," in *Plant stress physiology* (Wallingford: CABI), 94–117. doi:10.1079/9781845939953.0094.

Saito, S., Okamoto, M., Shinoda, S., Kushiro, T., Koshiba, T., Kamiya, Y., Hirai, N., Todoroki, Y., Sakata, K., Nambara, E., et al. (2006). A plant growth retardant, uniconazole, is a potent inhibitor of ABA catabolism in *Arabidopsis*. *Biosci. Biotechnol. Biochem.* 70, 1731–1739. doi:10.1271/bbb.60077.

Sasaki, H., Ichimura, K., Imada, S., and Yamaki, S. (2001). Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. *J. Plant Physiol.* 158, 847–852. doi:10.1078/0176-1617-00391.

Scott, I. M., Clarke, S. M., Wood, J. E., and Mur, L. A. J. (2004). Salicylate accumulation inhibits growth at chilling temperature in *Arabidopsis*. *Plant Physiol.* 135, 1040–1049. doi:10.1104/pp.104.041293.

Shi, Y., Tian, S., Hou, L., Huang, X., Zhang, X., Guo, H., and Yang, S. (2012). Ethylene Signaling Negatively Regulates Freezing Tolerance by Repressing Expression of *CBF* and Type-A *ARR* Genes in *Arabidopsis*. *Plant Cell* 24, 2578–2595. doi:10.1105/tpc.112.098640.

Shinkawa, R., Morishita, A., Amikura, K., Machida, R., Murakawa, H., Kuchitsu, K., and Ishikawa, M. (2013). Abscisic acid induced freezing tolerance in chilling-sensitive suspension cultures and seedlings of rice. *BMC Res. Notes* 6, 351. doi:10.1186/1756-0500-6-351.

Sin'kevich, M. S., Sabel'nikova, E. P., Deryabin, A. N., Astakhova, N. V., Dubinina, I. M., Burakhanova, E. A., and Trunova, T. I. (2008). The changes in invertase activity and the content of sugars in the course of adaptation of potato plants to hypothermia. *Russ. J. Plant Physiol.* 55, 449–454. doi:10.1134/S1021443708040031.

Sreenivasulu, N., Harshavardhan, V. T., Govind, G., Seiler, C., and Kohli, A. (2012). Contrapuntal role of ABA: Does it mediate stress tolerance or plant growth retardation under long-term drought stress? *Gene* 506, 265–273. doi:10.1016/j.gene.2012.06.076.

Strand, A., Hurry, V., Gustafsson, P., and Gardeström, P. (1997). Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. *Plant J.* 12, 605–14. doi:10.1046/j.1365-313X.1997.00605.x.

Taiz, L., and Zeiger, E. (2008). *Plant Physiology*. Fith Edition. Sinauer Associates Inc., Publishers

Sunderland, Massachusetts U.S.A.

Thomashow, M. F. (1999). Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 571–599. doi:10.1146/annurev.arplant.50.1.571.

Thomashow, M. F. (2001). So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* 125, 89–93.

Trouverie, J., Chateau-Joubert, S., Thévenot, C., Jacquemot, M.-P., and Prioul, J.-L. (2004). Regulation of vacuolar invertase by abscisic acid or glucose in leaves and roots from maize plantlets. *Planta* 219, 894–905. doi:10.1007/s00425-004-1289-3.

Trouverie, J., Thévenot, C., Rocher, J.-P., Sotta, B., and Prioul, J.-L. (2003). The role of abscisic acid in the response of a specific vacuolar invertase to water stress in the adult maize leaf. *J. Exp. Bot.* 54, 2177–86. doi:10.1093/jxb/erg234.

Usadel, B., Bläsing, O. E., Gibon, Y., Poree, F., Höhne, M., Günter, M., Trethewey, R., Kamlage, B., Poorter, H., and Stitt, M. (2008). Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. *Plant. Cell Environ.* 31, 518–547. doi:10.1111/j.1365-3040.2007.01763.x.

Vandenbussche, M. (2015). Petunia as a model. *Petunia Platf.* Available at: http://flower.ens-lyon.fr/PetuniaPlatform/Petunia_as_a_model.html [Accessed November 16, 2015].

Veisz, O., Galiba, G., and Sutka, J. (1996). Effect of abscisic acid on the cold hardiness of wheat seedlings. *J. Plant Physiol.* 149, 439–443. doi:10.1016/S0176-1617(96)80146-5.

Venema, J. H., Posthumus, F., and van Hasselt, P. R. (1999). Impact of suboptimal temperature on growth, photosynthesis, leaf pigments and carbohydrates of domestic and high-altitude wild *Lycopersicon* species. *J. Plant Physiol.* 155, 711–718. doi:10.1016/S0176-1617(99)80087-X.

Viswanathan, C., and Zhu, J.-K. (2002). Molecular genetic analysis of cold-regulated gene transcription. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 357, 877–86. doi:10.1098/rstb.2002.1076.

Walworth, A. E., Song, G., and Warner, R. M. (2014). Ectopic *AtCBF3* expression improves freezing tolerance and promotes compact growth habit in petunia. *Mol. Breed.* 33, 731–741. doi:10.1007/s11032-013-9989-7.

Walworth, A. E., and Warner, R. M. (2009). Differential cold acclimation ability of *Petunia spp*. *HortScience* 44, 1219–1222.

Wang, L., Zhao, C.-M., Wang, Y.-J., and Liu, J. (2005). Overexpression of chloroplast-localized small molecular heat-shock protein enhances chilling tolerance in tomato plant. *J. Plant Physiol. Mol. plant Biol.* 31, 167–74.

Warner, R. M. (2010). Temperature and photoperiod influence flowering and morphology of four *Petunia spp*. *HortScience* 45(3), 365–368.

Warner, R. M., and Walworth, A. E. (2010). Quantitative inheritance of crop timing traits in interspecific hybrid petunia populations and interactions with crop quality parameters. *J. Hered.* 101(3), 308–316. doi:10.1093/jhered/esp131.

Xia, J., Zhao, H., Liu, W., Li, L., and He, Y. (2009). Role of cytokinin and salicylic acid in plant growth at low temperatures. *Plant Growth Regul.* 57, 211–221. doi:10.1007/s10725-008-9338-8.

Yamori, W., Noguchi, K., Hikosaka, K., and Terashima, I. (2009). Cold-tolerant crop species have greater temperature homeostasis of leaf respiration and photosynthesis than cold-sensitive species. *Plant Cell Physiol.* 50, 203–215. doi:10.1093/pcp/pcn189.

Yamori, W., Noguchi, K., Hikosaka, K., and Terashima, I. (2010). Phenotypic plasticity in photosynthetic temperature acclimation among crop species with different cold tolerances. *Plant Physiol.* 152, 388–399. doi:10.1104/pp.109.145862.

Yang, Y.-W., Chen, H.-C., Jen, W.-F., Liu, L.-Y., and Chang, M.-C. (2015). Comparative transcriptome analysis of shoots and roots of TNG67 and TCN1 rice seedlings under cold stress and following subsequent recovery: insights into metabolic pathways, phytohormones, and transcription factors. *PLoS One* 10, e0131391. doi:10.1371/journal.pone.0131391.

Zhou, X., Wang, G., Sutoh, K., Zhu, J. K., and Zhang, W. (2008). Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim. Biophys. Acta - Gene Regul. Mech.* 1779, 780–788. doi:10.1016/j.bbagrm.2008.04.005.

6 Appendix

6.1 Supplementary Material for Chapter 2.2 (Transcriptome, carbohydrate, and phytohormone analysis of *Petunia hybrida* reveals a complex disturbance of plant functional integrity under mild chilling stress)

Table S1 | Expression data. (a) M-values, all expressed genes, (b) M-values, significantly regulated genes, (c) Expression values.

Due to the large amount of data, it was not possible to print the supplementary table S1 here. Please find the regarding file on the enclosed CD-ROM (file name: ***Chapter_2.2_Tab_S1.xlsx****).*

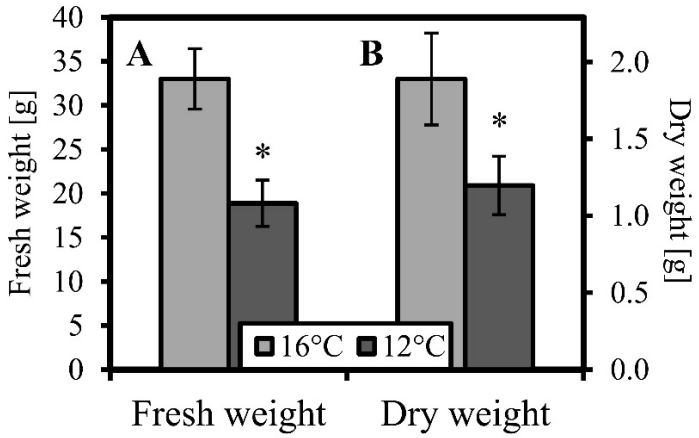


Figure S1 | Fresh and dry weight. Impact of sub-optimal temperature (black: 12.1 °C (day/night: 13.1 °C/11.1 °C), grey: 16.0 °C (16.8 °C/15.2 °C)) on fresh and dry weight over the period of 28 days. Shown are increases compared to 0 DoT. (A) fresh weight; (B) dry weight. (DoT = days after differentiation of temperature; greenhouse; n = 12; data are means +/- SE; asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

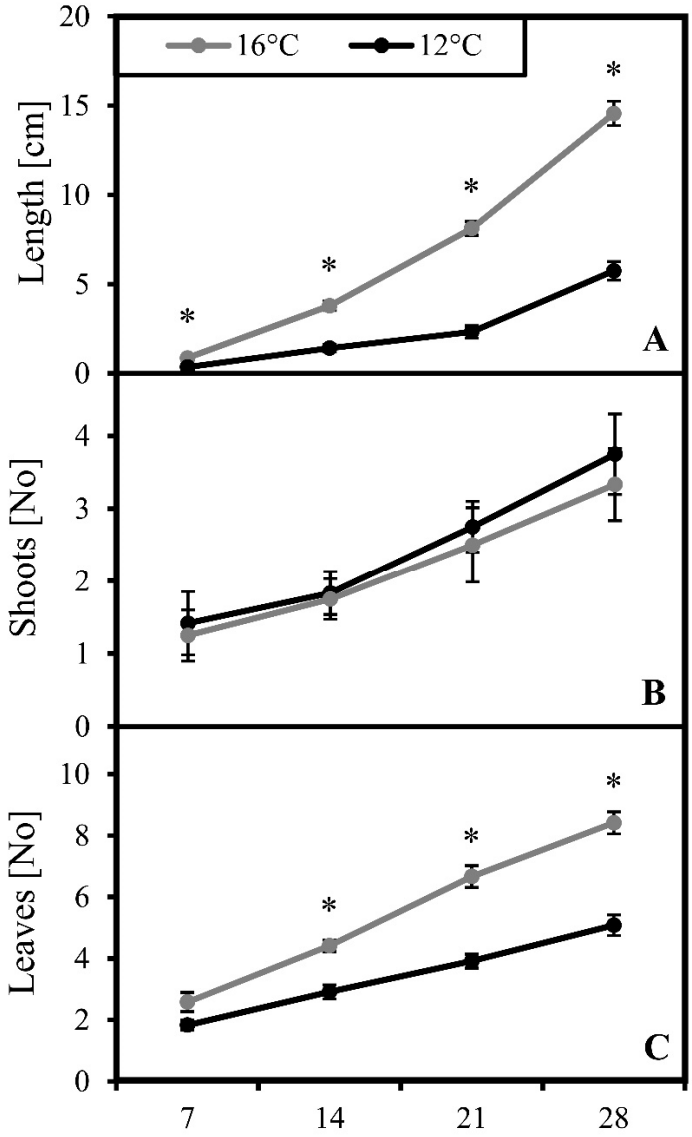


Figure S 2 | Growth parameters. Impact of sub-optimal temperature (black: 12.1 °C (day/night: 13.1 °C/11.1 °C), grey: 16.0 °C (16.8 °C/15.2 °C)) on different growth parameters over the period of 28 days. Shown are increases compared to 0 DoT. (A) length increase of the main shoot; (B) number of newly developed shoots; (C) number of newly developed leaves on the main shoot. (DoT = days after differentiation of temperature; greenhouse; $n = 12$; data are means +/- SE; asterisks indicate significant differences between temperature treatments for a given sampling time, $P \leq 0.05$).

6.2 Supplementary Material for Chapter 2.3 (Comparative analysis of two contrasting petunia cultivars indicates important functions of carbohydrate utilization and Abscisic acid in tolerance to mild chilling stress)

Due to the large amount of data, it was not possible to print the additional files 3-5 here. Please find the regarding files on the enclosed CD-ROM (file names: ***Chapter_2.3_Tab_S1A.xlsx, Chapter_2.3_Tab_S1B.xlsx, Chapter_2.3_Tab_S1C.xlsx****).*

Danksagung

An dieser Stelle bedanke ich mich von Herzen für die vielseitige Unterstützung, die diese Arbeit überhaupt erst ermöglicht hat:

Mein ganz besonderer Dank gilt Dr. Uwe Drüge für die Überlassung des Themas und die Möglichkeit, meine Promotion am Leibniz Institut für Gemüse- und Zierpflanzenbau (IGZ) in Erfurt anzufertigen. Darüber hinaus danke ich ihm für die herausragende wissenschaftliche Betreuung seitens des IGZ und die großartige Unterstützung im Laufe der vergangenen Jahre.

Ich danke Prof. Dr. Traud Winkelmann, die sich dazu bereit erklärt hat meine Arbeit seitens der Leibniz Universität Hannover (LUH) zu betreuen, für die hervorragende wissenschaftliche Betreuung, ihre fachliche Beratung sowie für die Organisation des WeGa-PhD-Programmes und die sehr gute Zusammenarbeit während meiner Zeit als WeGa-Doktorandensprecher.

Prof. Dr. Jutta Papenbrock und Prof. Dr. Thomas Debener (beide LUH) danke ich für die Mitwirkung in der Prüfungskommission.

Prof. Dr. Philipp Franken danke ich besonders für die wissenschaftliche Beratung beim zweiten und dritten Paper, v. a. bzgl. der Array-Auswertung. Ein großer Dank geht an alle, die mich direkt oder indirekt bei der Arbeit unterstützt haben. Ganz besonders an Sabine Czekalla und Bärbel Broszies, die eine unverzichtbare Hilfe im Gewächshaus und Labor waren. Klaus Fricke unterstützte mich tatkräftig bei der Durchführung der Phytohormonanalyse, Kerstin Fischer bei der Extraktion der RNA für die Microarray-Studien und Kathrin Schultz bei den Substratanalysen. Dr. Siegfried Zerche danke ich für die Düngeempfehlungen, statistische Beratungen und die anregenden wissenschaftlichen Diskussionen. Vielen Dank an Dr. Roland Kadner und an die Gärtnerinnen des IGZ für die Unterstützung bei der Pflanzenkultur in Gewächshaus und Klimakammer. Ich danke Dr. Yvonne Stonek für ihren wissenschaftlichen Rat und viele interessante Gespräche. Danke auch an Rita Zrenner und die übrigen Wissenschaftler des IGZ Erfurt für den wissenschaftlichen Austausch. Dank gilt auch Jörg Krüger, der stets eine Lösung für alle technischen Probleme am PC fand. Ich danke außerdem Tobias Döring und Achim Jentsch für ihre stets schnelle und tatkräftige Hilfe bei allen übrigen technischen Problemen, die mir darüber hinaus mit ihrer unkomplizierten und erfrischenden Art stets ein Lichtblick waren.

Vielen Dank an die Kooperationspartner des WeGa-Verbunds 5 für den regelmäßigen wissenschaftlichen Austausch über die gesamte Laufzeit meines Projektes hinweg: Insbesondere danken möchte ich hier Annika Langhans (LUH) deren Screening-Daten ich dankenswerter Weise in das erste Kapitel miteinbeziehen durfte; Prof. Dr. Heiko Mibus-Schoppe (Hochschule Geisenheim) für den fachlichen Austausch bei der Erstellung des Sortenscreening Papers; Dr. Susanne Amberger-

Ochsenbauer und Florian Steinbacher (Weihenstephan-Triesdorf University of Applied Sciences, Freising) für die Durchführung der Photosynthese-Messungen; Dr. Johanna Knopp und Dr. Henning von Alten (LUH) für den wissenschaftlichen Austausch; Dr. Beate ter Hell und Dr. Dirk Ludolph (Lehr- und Versuchsanstalt für Gartenbau Ahlem) für ihre Ratschläge bezüglich der Kulturbedingungen und Selecta Klemm GmbH & Co. KG (Stuttgart) für die Sorten-Empfehlungen und die Bereitstellung von bewurzelten Stecklingen.

Ich danke auch Dr. Mohammad-Reza Hajirezaei, der mir die Möglichkeit geboten hat, in seinem Labor am IPK Gatersleben verschiedene Enzymanalysen zu erlernen. Prof. Dr. Edgar Maiss (LUH) danke ich für die umfassende Hilfe bei der Virusdetektion. Weiterhin gilt mein Dank Herbert Geyer (LUH) für das zur Verfügung gestellte Pflanzenmaterial. Darüber hinaus danke ich den Doktoranden des WeGa-PhD-Programmes für die schöne gemeinsame Zeit und den regelmäßigen Austausch.

Ein besonders großer Dank gilt meinen lieben Eltern, ohne deren uneingeschränkte Unterstützung Studium und Dissertation in den letzten zwölf Jahren so nicht möglich gewesen wären. Ganz besonders bedanken für die moralische Unterstützung und die Freundschaft durch die Studien- und Promotionszeit möchte ich mich bei Annett, meiner Promotions- und Bürokollegin am IGZ, bei Marlen, Natalie und Viktoria, und natürlich bei meiner lieben Freundin Mady.

Funding

The work was funded by the BMBF (Federal Ministry of Education and Research of the Federal Republic of Germany) within the BMBF-AgroClustEr: ‘WeGa - Horticulture Research Network - Product and Production Safety in Intensive Plant Production Systems’ and further supported by ministries of the state of Brandenburg, the Free State of Thuringia and the Federal Republic of Germany.

Diese Arbeit wurde im Rahmen des AgroClustErs WeGa, des Verbundprojektes „Terminproduktion von Zierpflanzen in Gewächshäusern (Kühletoleranz)“ angefertigt. Die finanzielle Förderung erfolgte durch das BMBF (Bundesministerium für Bildung und Forschung der Bundesrepublik Deutschland) und durch Ministerien des Landes Brandenburg, den Freistaat Thüringen und der Bundesrepublik Deutschland.