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Studies on Water-Soluble Carbohydrates in Wheat (*Triticum aestivum* L.): Regulating Traits, Model Analysis, Early Chilling Effects, and Future Perspectives

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Declaration

I hereby declares that the thesis entitled "**Studies on water-soluble carbohydrates in wheat (*Triticum aestivum* L.): Regulating traits, model analysis, early chilling effects and future perspectives**" has been carried out in the Institute for Crop Production and Grassland Research, University of Hohenheim, Stuttgart, Germany under the guidance of Prof. Dr. Wilhelm Claupein. The work is original and has not been submitted in part or full by me for any degree or diploma at any other University.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

(**Ravi Valluru**)

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Table of Contents

List of Tables

List of Figures

tonoplast and released as single-membrane vesicles into the cytosol. F. The released single-membrane vesicles may move to the PM with the help of cytoskeleton structures and fuse with the membrane. After discharge, vesicles may integrate with the membrane (see inset, F1, F2 for magnified vesicle bilayer and its fusion with PM and releasing solutes). The released fructans may be selectively trimmed by apoplastic FEHs, and the resulting sugars may interact with PM lipids for membrane stabilization. V, vacuole; CS, cytoskeleton structures

22 Simple representation (not docking) of 1-kestose (light blue); 1,1 nystose (yellow); sucrose (grey) and fructose (pink) inserted between the headgroups of phosphatidylcholine membrane lipids (phosphate: orange; carbon: green; nitrogen: blue; oxygen: red). Coordinates of 1,1 nystose and the membrane lipids were calculated using molecular dynamics (Timmermans *et al.*, 1993; Heller *et al.*, 1993). Picture was drawn with Pymol (Delano, 2002) 110

Appendices

Abbreviations

σ_g^2	Genetic variance
σ_e^2	Residual variance
%	Percent
µmol	Micromol
1-FFT	Fructan: fructan 1-fructosyltransferase
1-SST	Sucrose: sucrose 1-fructosyltransferase
6-SFT	Sucrose: fructan 1-fructosyltransferase
ACC	Available carbon for carbohydrates
ADP	Adenosine diphosphate
AGPase	ADP-glucose pyrophosphorylase
A_i	Chemical substances
ANCOVA	Analysis of covariance
APN	Actual plant nitrogen concentration
asl	Above sea level
C	Carbon
CGR	Crop growth rate
Chl	Chlorophyll content
Chl_{LA}	Chlorophyll per unit leaf area
C_i	Cell-wall polysaccharides
C_L	Cellulose content
C_{LA}	Carbon per unit leaf area
CO_2	Carbondioxide
CPC	Critical plant nitrogen concentration
CT	Chilling temperature
DP	Degree of polymerization
F	Steady-state fluorescence yield
FEHs	Fructan exohydrolases
FL_A	Flag-leaf area
FL_L	Flag-leaf length
FL_W	Flag-leaf width
Fm	Maximum fluorescence yield
Fm^I	Light adapted state yield
Fo	Dark fluorescence yield

FT	Flowering time
FTs	Fructosyltransferases
GDD	Growing degree days
GSI	Genotypic sensitive index
H^2	Broad-sense heritability
H_C	Hemicellulose content
H_p	Plant height
HPLC	High performance liquid chromatography
IHO	IhingerHof Research Station
J cm^{-2}	Joules per square centimeter
J_L	Non-cyclic electron flow
JO_2	Rate of oxygen evolution
K	Light extinction coefficient
K	Potassium
$kgha^{-1}$	Kilogram per hectare
l	Leaf
LAI	Leaf area index
LMR	Leaf mass ratio
L_N	Leaf nitrogen concentration
LN	Leaf number
m ha	Million hectare
mt	Million tonn
m^2	Square meter
MANOVA	Multivariate analysis of variance
mg g^{-1}	Milligram per gram
mm	Millimeter
mM	Millimolar
MPC	Minimum plant nitrogen concentration
N	Nitrogen
NAR	Net assimilation rate
NCT	Non-chilling temperature
N_{LA}	Nitrogen per unit leaf area
NPQ	Nonphotochemical quenching
NSCs	Non-structural carbohydrates
^oC	Degrees Celsius
Oi	Observed values
OLH	ObererlindenHof

P	Phosphorus
PAM	Pulse amplitude modulation
Pi	Predicted values
P_L	Peduncle length
PPFD	Photosynthetic photon flux density
qP	Photochemical quenching
QTL	Quantitative trait loci
r	Root
RGR	Relative growth rate
RI	Refractive index
RMR	Root mass ratio
RMSE	Root mean square error
RS_R	Root: shoot ratio
RUE	Radiation use efficiency
RWSC	Rate of WSCC accumulation
S	Selection differential (total selection)
s	Stem
S^{-1}	Per second
S_i	C_6 sugars
SLA	Specific leaf area
SMR	Specific mass ratio
S_N	Stem nitrogen concentration
t ha^{-1}	Tonn per hectare
Tg	Devitrification temperature
tm	Transition temperature
T_N	Number of tillers
TVE	Tonoplast-vesicle-derived exocytosis
v/v	Volume by volume
V_{Mass}	Vegetative biomass
VMM	Vesicle-mediated mechanism
W_i	Dry weight
WSCs	Water-soluble carbohydrates
B	Selection gradient (direct selection)
σ	Ratio of relative masses of C to oxygen and hydrogen
ΦCO_2	Quantum yield of carbondioxide assimilation
ΦCO_2/ ΦPSII	Ratio of ΦCO_2/ ΦPSII

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dedicated to my beloved family

The greatest service which can be rendered to any country is to add an useful plant to its culture; especially a 'bread grain'.

Thomas Jefferson, 1821

Chapter One
GENERAL INTRODUCTION

General Introduction

Cereals, since the beginning of agriculture ~10 000 years ago, have provided the main source of calories for mankind. Of these, wheat, a major member of the tribe Triticeae[1], has particularly served as the principal grain stock enabling the founding of agriculture in the Middle East and led to its successful spread around the world (Zohary & Hopf, 2000).

[1]Triticeae is a tribe within the Poaceae family of grasses that includes genera with many domesticated species, including wheat, has some of the most complex genetic histories.

'Wheat' is used to describe several related grain crops. The most important wheat species grown today are bread wheat (*Triticum aestivum*) and pasta wheat (*T. turgidum*) (Appendix 1). A third species, einkorn wheat (*T. monococcum* L.), has great historic but very little agricultural significance now. Besides einkorn, several wheats and its relatives had been evolved, which share the same basic set of seven chromosomes, mostly in diploid form, and are given a 'genomic constitution' classification according to their meiotic pairing characteristics in diploid hybrids.

The first evolutionary event leading to polyploid wheats was hybridization of diploid wheat closely related to *T. urartu* (genomic AA) with a yet unknown species from the *Sitopsis* section that provided the B genome and was closely related to *Aegilops speltoides* (SS) (Appendix 2; Biswas *et al.*, 2008). Eventually, this fertile tetraploid (AABB) was domesticated as emmer wheat, or *T. turgidum*. Although most are of little economic significance now, one subspecies, *T. turgidum* ssp. *durum*, which gave rise to the pasta wheat cultivars of today (Biswas *et al.*, 2008), is still grown widely. A second evolutionary event that led to the bread wheat lineage occurred when tetraploid emmer wheat crossed with *A. tauschii*, a wild diploid species with a DD genome (Appendix 2). As with the hybridization event that generated *T. turgidum,* this, resulted in chromosome doubling in gametes, and progeny that gave rise to a fertile, hexaploid species with an AABBDD genome—a species now known as *T. aestivum* or bread wheat.

[2]Despite the inclusion of positive characters, D genome seems to reduce photosynthesis particularly under stress conditions, for example under ozone stress (Biswas *et al.*, 2008)

Because the D genome[2] of bread wheat was originated from *A. tauschii*, it carried genes and alleles adapted to the more continental climate of central Asia,

thus enabling *Triticum aestivum* L. to be cultivated widely, both geographically and environmentally, than emmer wheat. Significantly, the D genome encoded proteins that restored the softness of the grain endosperm (Chantret *et al.*, 2005), and thereby improved bread-making properties. It also contains proteins that trap CO_2 during yeast fermentation. Combined, these factors led to the widespread cultivation of bread wheat around the world and to the development of several different subspecies. As such, hexaploid bread wheats account for ~90% of world wheat production today (Table 1, FAO, 2009).

Table 1 Global production and yield of cereal species belonging to *Triticeae.**

Species	Production (m t)	Area (m ha)	Yield (tha^{-1})	Percent total cereal production
Wheat	619	213	2.9	28
Barley	137	53	2.6	6.1
Rye	15	7	2.2	0.7
Triticale	13	3.7	3.5	0.6
Total Triticeae	784	273	2.8	35

*Data from Food and Agriculture Organization of the United Nations, FAO, July 2007 (www.faostat.fao.org/).

1.1 Grain filling: life-driven reproductive process

A single wheat grain, on an average, contains several units of water (12%), protein (12%), mineral (1.8%), fat (2%), crude fibre (2.2%) and carbohydrates[1] (70%) (Palmer *et al.*, 2001). This wide nutritional quality allow wheat grain flour to be used in versatile ways such as flat and steamed breads; cookies, cakes, breakfast cereal, pasta, juice, noodles, couscous and for fermentation to make beer, alcohol, and vodka or biofuel. However, the principal components of wheat grain are gluten and starch. Gluten[2] is an important nutritional protein present in wheat grain; while starch[3] is the major carbohydrate portion. Much of the starch, however, signifies a converted fraction of fructan[4], another major reserve carbohydrate, stored in the vegetative portions of the plant.

Grain yield of cereals is a complex physiological trait, and symbolize the final stage of growth where fertilized ovaries develop into caryopses. The duration and rate of grain filling determines the final grain weight, one of the key components of total yield. Typically, grain filling[5] is described by a

[1]Carbohydrates, are sugars and their polymers, which serve as important structural elements in cells, and are primary sources of chemical energy for living systems.

[2]Gluten is an insoluble mixture of the proteins gliadin and glutenin. These exit, together with starch, in the endosperms of grains.

[3]Starch is a polysaccharide carbohydrate formed by several glucose units and stored in the endosperms of grains.

[4]Fructan, a polymer of fructose, is the third major reserve carbohydrate found in 15% of flowering species, particularly in temperate cereals and grasses.

[5]Grain filling is broadly divided into two phases: grain enlargement and grain filling.

sigmoid curve and thus has three phases: a short lag phase, a long linear phase, and a plateau. The linear growth phase is fundamentally important than total duration as this dumps a large portion of carbohydrate and protein complexes into the developing grains (Ehdaie *et al.*, 2008). Fast and synchronized filling[1] is associated with higher yields and higher percentage of filled grains (Yang *et al.*, 2000). This phase of grain filling is fairly regulated either environmentally or genetically (Yoshida, 1972; Yang & Zhang, 2006). For example, water stress occurring during early grain development curtails the kernel sink potential by reducing the number of endosperm cells and amyloplasts formed (Saini & Westgate, 2000), thus reducing grain weight as a result of a reduction in the capacity of the endosperm to accumulate starch, in terms of both rate and duration (Yoshida, 1972).

[1]Genetically, there are two grain-filling patterns, synchronized and asynchronized. In synchronized filling, both superior (top of panicle) and inferior (base of panicle) spikelets reach rapid grain filling period at similar dates; while at different times in asynchronized filling.

In cereals, carbons from two sources contribute to grain filling: current assimilates transferred directly to the grain and assimilates redistributed from reserve pools in vegetative tissues either at pre or post-anthesis (Schnyder, 1993). Reserve pools provide the substrate needed to maintain transport and supply of assimilate to grains during the dark period of the diurnal cycle and also during the later grain-filling period, when the photosynthetic apparatus is senescencing and the rate of dry matter accumulation of grains exceeds the rate of dry matter accumulation of the total crop (Schnyder, 1993). In ample moisture situations, pre-anthesis assimilate reserves in the stems and sheaths of wheat (*T. aestivum*) contribute 10-40% of the final grain weight (Schnyder, 1993; Gebbing & Schnyder, 1999; Yang & Zhang, 2006). However, reserve mobilization to the grain becomes crucial when plants are subjected to terminal water stress or if the yield potential is largely based on high biomass accumulation (Yoshida, 1972; Plaut *et al.*, 2004; Ruuska *et al.*, 2006, 2008; Xue *et al.*, 2009).

[2]Senesence is nothing but dying of plant tissue, indeed cell, which trigger redistribution of nitrogen and carbon complexes to the neighboring tissues, and strong alternative sinks such as ears.

Grain-filling is closely linked to the whole-plant senescence[2] process (Yang *et al.*, 2000; Mi *et al.*, 2002). When senescence is delayed unfavourably, which usually occurs in case of heavy use of nitrogen (N) fertilizer or adoption of lodging-resistant cultivars that stay 'green' for too long (i.e. plants remain green when grains are due to ripen), results in a low grain-filling rate, leading to many poorly filled grains (Yang & Zhang, 2006). Drought stress at grain filling induces early senescence and shortens the grain filling period but increases remobilization of assimilates from the straw to the grains (Yoshida, 1972; Kobata & Takami, 1981; Nicolas *et al.*, 1985; Palta *et al.*, 1994; Ehdaie & Waines, 1996; Asseng & van Herwaarden, 2003; Plaut*et al.*, 2004). However, if the senescence is functionally delayed when photosynthesis and phloem translocations are still functional, for example by favourable weather conditions, then such delayed

senescence may help achieve higher dry mass production and possibly higher grain yield (Thomas & Smart, 1993). In many cases, kernels and their connecting rachis or rachilla, however, seem to senesce earlier than the stem and leaves, leaving much unused WSCs left in the stem and sheath (Liang *et al.*, 1994; Ricciardi & Stelluti, 1995; Yang *et al.*, 2002).

Grain filling, on the sink side, is a process of active metabolism of carbohydrate and starch accumulation in kernels. Sucrose in the grains is cleaved by sucrose synthase (Fisher & Gifford, 1986). This process yields glucose and fructose, which is thought to be the first step in sucrose to starch conversion in the grains. However, its activity is considered to be linked to sink strength in the developing cereal grain (Sun *et al.*, 1992; Wang *et al.*, 1993; Kato, 1995).

1.2 Water-soluble carbohydrates: more than reserve carbohydrates

Plants synthesize carbohydrates (hexoses) through photosynthesis. These hexose sugars are used for metabolic maintenance, growth and development and generation of new plant tissues. Excess carbohydrates, after meeting internal metabolic demands, store in vegetative parts, are known as reserve carbohydrates[1] or water soluble carbohydrates (WSCs) or non-structural carbohydrates (NSCs) (Judel & Mengel, 1982; Blacklow *et al.*, 1984). Differences in the accumulation of WSCs in stems among genotypes could potentially result from various factors such as photosynthetic capacity, carbon use efficiency and carbon partitioning between stem reserve deposition and other physiological processes (e.g. maintenance of respiration, growth and cell wall synthesis) (Xue *et al.*, 2008).

In wheat, the main storage forms of WSCs are a range of fructo-oligosaccharides (fructans[2]), as well as sucrose and hexoses (Hendrix *et al.*, 1986; Kühbauch & Thome, 1989; Wardlaw & Willenbrink, 1994; Ruuska *et al.*, 2006; Yang & Zhang, 2006; Xue *et al.*, 2008). At the stage of maximum WSCs, fructans and sucrose represented 85% and 10%, respectively, of the total WSCs in wheat stem internodes (Blacklow *et al.*, 1984). This storage peaks well into the period of grain filling under adequate moisture conditions and declines during the later stages of kernel development to supply a high proportion of assimilates needed for concurrent kernel development (Wardlaw & Willenbrink, 1994).

In general, long-term storage of fructans occurs in heterotrophic sink tissues, which are dependent on the import of carbohydrates, such as in the

[1]Reserve carbohydrates vary between plant species. By far and large, starch is the major reserve carbohydrate followed by sucrose. Fructan is the third major reserve carbohydrate.

[2]Fructans are acid-labile, water soluble carbohydrates. Among two major types, inulins and levans, inulins are more flexible than levans, and thus more active in membrane protection under abiotic stresses.

tubers of Jerusalem artichoke (Kaeser, 1983), the root of chicory (Van den Ende & Van Laere, 1996), or in the internodes of wheat (Bonnett & Incoll, 1993; Gebbing, 2003). Significant fructan accumulation can also be found in growing tissues of grasses such as leaf sheaths, tiller bases, stems, elongating leaf bases, and wheat grains (Schnyder, 1993), which are also tissues of relatively low photosynthetic activity. However, the duration available for each internode (internode elongation period) may determine reserve pools in the plant (Ehdaie *et al.*, 2006) since reserve pools in different internodes commence at different times but cease at about the same time (Schnyder, 1993).

The predominant role for fructans is to bridge the temporal gaps between resource availability and demands. However, they can also fuel rapid regrowth in grasses (Morvan-Bertrand *et al.*, 2001); acts as osmolytes (Kawakami *et al.*, 2008); regulate osmosis during flower opening (Le Roy *et al.*, 2007), and protect plants against cold and drought stress through membrane stabilization (Hincha *et al.*, 2003; Valluru *et al.*, 2008). Further, fructan synthesis is assumed to be a mechanism to sustain sucrose gradients and thus control carbon metabolism in leaves (Cairns, 2003). In addition, inulin-type fructans have gained importance as functional food ingredients (Roberfroid, 2007).

Fructans are believed to be synthesized from sucrose in the central vacuole of plants (Frehner *et al.*, 1984). Plant fructans can be divided into five classes (inulin, levan, mixed levan–graminan, inulin neoseries and levan neoseries). Dicotyledonous species accumulate mostly inulin fructan series (Van den Ende & Van Laere 2007), whereas monocotyledonous species predominantly store a mixture of the different fructan types (Livingston *et al.* 1993, Luscher & Nelson 1995) (Appendix 3).

Fructans are synthesized by the action of two or more different fructosyltransferases[1] (FTs) (Edelman & Jefford, 1968; Van den Ende & Van Laere, 1996). In dicotyledonous species, the combination of sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) produces the inulin series. In the monocotyledonous Poaceae family, sucrose:fructan 6-fructosyltransferase (6-SFT) plays an important role in fructan synthesis. Fructan breakdown is accomplished by fructan exohydrolases (FEHs), leading to the release of fructose which, in turn, has to be converted to the precursors required for the re-synthesis of sucrose (Huber & Huber, 1996). Indeed, Yang *et al.* (2004) found that FEH activities were substantially enhanced under water stress during the grain-filling period and positively correlated with the total WSCs and fructan remobilization from wheat stems.

[1]Fructosyltransferases (FTs) are fructan synthesizing enzymes.

Next to its yield-driven role, fructan metabolism have been shown to be closely related to frost and drought tolerance (Trunova, 1965; Pontis, 1989; Tognetti *et al.*, 1990; Yukawa and Watanabe, 1991; Santoani *et al.*, 1993; Yoshida *et al.*, 1998; De Roover *et al.*, 2000), because fructan-accumulating species are more prominent in cold and dry environments and absent in tropical and aquatic environments (Hendry, 1993). Indeed, fructans directly interact with membranes, making a direct hydrogen bond with the phosphate groups and thus protect them under stresses (Hincha *et al.*, 2000) (Appendix 4). Levan-type fructans preferentially interact with small-headgroup lipids. Inulin-type fructans show a deep interaction with lipids (Vereyken *et al.*, 2003). Additionally, inulins showed a lower crystallization rate[1] above the devitrification temperature[2] so that they provide better protection of membranes (Vereyken *et al.*, 2003). These studies strongly support fructans as protective agents under abiotic stresses.

Fructan accumulation is enhanced by conditions that increase photosynthesis (i.e. long photoperiods) while decreasing the demand for carbon (i.e. low temperatures, low nitrogen availability, and drought) (Hendry, 1993). The effect of temperature on fructan metabolism, accumulation, and partitioning has been widely reported (Chatterton *et al.*, 1989; Prud'Homme *et al.*, 1992). Other reports include the effects of drought stress (Pilon-Smits *et al.*, 1995; 1999), hypoxia (Albrecht *et al.*, 1997; 2004), carbon dioxide (Smart *et al.*, 1994), photoperiod (Legnani & Miller, 2001), phosphate (Morcuende *et al.*, 2005) and nitrate concentration (Améziane *et al.*, 1995, 1997; van Herwaarden *et al.*, 1998, 2003; Morcuende *et al.*, 2004; Ruuska *et al.*, 2008).

Nitrogen (N) assimilatory processes are closely related to carbon (C) assimilation. Therefore, the disruption in the supply of either of the resources can impose marked restrictions on the assimilation of the other (Lattanzi *et al.*, 2005; Bertheloot *et al.*, 2008). In suggesting a causal relationship between N and carbohydrate assimilation, two distinct trends are commonly found: (a) a negative signalling between N and carbohydrate assimilation (van Herwaarden *et al.*, 1998, 2003; Morcuende *et al.*, 2004; Ruuska *et al.*, 2008); (b) the translocation of a large portion of carbohydrates to the root system under N stress (Rufty *et al.*, 1988; Dodd *et al.*, 2002; Cruz *et al.*, 2003).

During nitrate assimilation, carbohydrate synthesis is decreased as more carbon is converted to phosphoenolpyruvate through glycolysis[3] (Stitt *et al.*, 2002). Nitrate induces the expression of the regulatory subunit of ADP-glucose pyrophosphorylase and increase starch and sucrose synthesis (Ruuska *et al.*, 2008). Consistently, nitrate stress is a negative signal, rapidly inducing fructan

[1]Removal of water molecules between lipid membranes under moisture stress brings lipid headgroups closer. This, in turn, increase liquid crystalline phase transition temperature mixing lipids together and resulting in more water loss through cell walls.

[2]Devitrification temperature (Tg) is the temperature at which a carbohydrate becomes brittle on cooling or soft on heating.

[3]Glycolysis is the metabolic pathway that converts glucose into pyruvate and the resultant energy used for the generation of ATP.

accumulation together with an induction of FTs (Wang & Tillberg, 1996; Van den Ende *et al.*, 1999; Morcuende *et al.*, 2004; Shiomi *et al.*, 2006; Ruuska *et al.*, 2008). The enhanced FTs may regulate fructan accumulation either directly or via downstream effects on the plant growth or metabolism under N stress (Wang *et al.*, 2000). Further, N deficiency elevated the transcripts for a number of protein or sugar kinases associated with the regulation of C storage in wheat (Ruuska *et al.*, 2008).

1.3 Modeling water-soluble carbohydrate accumulation

Crop modeling has been an emerging feature of agronomic research for more than three decades (Sinclair & Seligman, 1996). Crop models play a heuristic role in scientific investigation, crop management decisions, education, and policy issues concerning agricultural lands. In addition, an increasing role for models was established in the understanding of crop genetic regulation and anticipated responses to genetic alterations. Modeling crop traits, which are quantitative and complex in nature, is essential as the genes identified for one or a few of these yield components have a strong significance, in one way or another, to increase crop yield.

Previously, crop growth and development and grain protein concentration have been reasonably simulated by wheat modelers (Porter, 1993; Jamieson & Semenov, 2000; Asseng *et al.*, 2002; Brisson *et al.*, 2002). Most of these models also simulate grain size (grain dry mass). Later, attempts were made to model grain protein composition (Martre *et al.*, 2003)[1]. Such efforts should be pursued, in particular by modeling the aggregation of the gluten forming proteins. The genetic determinism (allelism) of storage proteins is well known (Branlard *et al.*, 2001, 2003) and integration of some genetic parameters influencing wheat protein composition can now be operated for a broader use of simulation models (Charmet *et al.*, 2005).

[1]However, no genetic parameters (allelic variants) can be advantageously introduced in modeling grain size or protein concentration, largely due to lack of mechanistic description in the current models.

However, many other grain components, besides proteins, are involved in different aspects of wheat quality. These include starch (size distribution of starch granule, percentage of damaged starch, amylose to amylopectine ratio), fibres (arabinoxylanes and glucans), lipids, minerals, and vitamins. These grain components or their respective precursors, particularly the compounds which appear early in growth and convert into starch granules later such as sucrose and fructans, might be most interesting for crop modelers.

In summary, WSCs stored in the vegetative portions of the plant during pre-anthesis period are crucial compounds in regulating grain yields as well as crop adaptation to adverse environmental conditions. These reserves are fairly regulated by several external crop management factors such as soil nitrogen availability. Simulating such nitrogen effects on WSC accumulation could majorly aggregate percent of WSCs incorporation in the grain.

1.4 Objectives of the thesis

Considering these untouched research areas of WSCs, the present thesis was composed of four broad objectives:

1. Identifying several morphological and physiological traits regulating water-soluble carbohydrates in wheat under three nitrogen levels;

2. Developing simulation model for water-soluble carbohydrate accumulation under three nitrogen levels;

3. Quantifying the effects of early chilling stress on water-soluble carbohydrates during recovery period and;

4. Identifying and warranting interesting future research areas for WSCs.

All space and matter, organic or inorganic, has some degree of life in it, but its structure and arrangement can signify whether matter/space is more alive or less alive.

Christopher Alexander 1936

Chapter Two
STRUCTURE OF THE THESIS

Thesis structure

The present thesis is designed with eight chapters each dealing with a specific set of research questions by providing the basic information on the topic, and its significance in the context of agronomic research. Specifically, major chapters include Trait regulation (Chapter 3), Model analysis (Chapter 4), early chilling effects (Chapter 5), and future perspective (Chapter 6). In addition, general introduction (Chapter 1), general discussion (Chapter 7) and summary (Chapter 8) are included.

Chapter 1 describes the general information on the topics of the thesis. It provides the basic knowledge on the respective topics for understanding the overall objectives of the thesis.

Chapter 2 describes the organization and arrangement of the topics in the thesis.

Chapter 3 provides morpho-physiological traits regulating water-soluble carbohydrates under three nitrogen levels. It contains the article ' Valluru R, Link J, Graeff S, Claupein W. Morpho-physiological traits and regulation of reserve carbohydrates in wheat under three nitrogen levels' submitted to New Phytologist, June 2009. This article explains that total WSCs were primarily associated with five traits, vegetative biomass, flag-leaf width, root: shoot ratio, leaf N concentration, and radiation use efficiency in all nitrogen levels.

Chapter 4 describes the model analysis for water-soluble carbohydrate accumulation in wheat under three nitrogen levels. It contains the article 'Valluru R, Link J, Claupein W. Simulation modeling of water-soluble carbohydrates accumulation in wheat under three nitrogen levels' to be submitted. A simulation model for WSCs accumulation was developed and fitted into a non-linear phenological model.

Chapter 5 describes early chilling effects on water-soluble carbohydrates in two primitive wheat species. It contains the article "Valluru R, Link J, Claupein W. Consequences of early chilling effects in two primitive *Triticum* species' submitted a revised version to Plant Biology in April 2009. Early chilling stress resulted in significantly lower WSCs at flowering in both species, also confirmed from the phenotypic selection analysis, which favoured lower WSCs under early chilling. Moreover, a significant cost of plasticity was found for fructans under early chilling environment.

Chapter 6 provides information on potential future perspective for fructan, the major water-soluble carbohydrate. It contains the article "Valluru R, Lammens W, Claupein W, Van den Ende W. 2008. Freezing tolerance by vesicle-mediated fructan transport' published in Trends in Plant Science, August 2008. This opinion explains how fructans were transported from the site of synthesis (vacuole) to the site of action (cellular membranes) to protect membranes under abiotic stresses, and proposed a unique vesicle-mediated model.

Chapter 7 provides a general discussion of all the thesis chapters, their significance and practical application for further exploitation.

Chapter 8 explains the overall objectives of the thesis, together with their experimental results and discussions on the name of Summary.

We may conclude that whatever part or character is most valued - whether the leaves, stems, tubers, bulbs, flowers, fruits or seed of plants…that character will almost invariably be found to present the greatest amount of difference both in kind and degree.

Charles Darwin, 1868

Chapter Three

TRAITS REGULATING WATER-SOLUBLE CARBOHYDRATE ACCUMULATION

This paper will appear as

Ravi Valluru, Johanna Link, Simone Graeff, Wilhelm Claupein. Morpho-physiological traits and regulation of reserve carbohydrates in wheat under different nitrogen levels. (Submitted, June 2009)

Traits Regulating Water-soluble Carbohydrate Accumulation

3.1 Abstract

Keywords

Morpho-physiological traits,
nitrogen,
selection analysis,
water-soluble carbohydrates,
wheat

Improving water-soluble carbohydrates (WSCs), a highly heritable and yield-driven agronomic trait, is essential for sustaining crop yields as well as crop adaptation to abiotic stresses. Marker-aided selection to improve WSCs in wheat has not been substantial; perhaps partly due to the small affects of multiple WSCs quantitative trait loci. However, the selection for superior segregants can be accelerated using rapidly measured traits. In this study, we investigated 33 morpho-physiological traits regulating WSCs in eight wheat genotypes under three nitrogen (N) levels. 26 investigated traits were significantly, positively or negatively, correlated with total WSCs in all N levels, and 20 of them were consistent across N levels. There was significant variation in WSCs between N levels with wide broad sense heritability. Among all traits, root: shoot ratio (RS_R), stem nitrogen (S_N), leaf nitrogen (L_N) and nitrogen per unit leaf area (N_{LA}) were negatively correlated with total WSCs. This suggests that plant N is a negative signal for total WSCs; indeed, a unit increase in leaf N resulted in 28% decrease in total WSCs. Vegetative traits displayed differential regulating effects across N levels to maximize WSCs: (1) an increase in vegetative biomass (V_{MASS}) was selected for under low and optimum N, but not under high N; (2) more flag-leaf width (FL_W) was favoured under low N, but it had only a small effect on WSCs under high N and; (3) a higher resource allocation to roots was selected for under high N, but selected against optimum and low N. However, vegetative traits, together with physiological traits, operated interactively to maximize total WSCs. In all N situations, the large effects of radiation use efficiency (RUE), together with specific morphological adaptations, in general appeared to buffer the negative effects of N on WSCs. Interestingly, the negative correlation between WSCs and N seemed to alter the positive association between WSCs and V_{MASS}, reinforcing an argument against the positive norm of WSCs and V_{MASS}. Yet, a specific combination of trait correlations, rather than a single trait, appeared to evolve under N-specific selection rendering the basis for maximizing WSCs in wheat.

3.2 Introduction

Many temperate plants, including wheat (*Triticum* species), store excess carbons in the form of water-soluble carbohydrates (WSCs), primarily consisting of a range of fructo-oligosaccharides (fructans), as well as sucrose and hexoses (Ruuska *et al.*, 2006; Yang and Zhang, 2006; Xue *et al.*, 2008). Because they store at high levels (more than 40% of total stem dry weight in wheat) and are rapidly remobilized, WSCs were implicated as the dominant carbon source for mobilization to the grains, particularly when active photosynthesis was inhibited by terminal drought stress in cereals (Plaut *et al* 2004; Takai *et al.*, 2006; Foulkes *et al.*, 2007). In addition, WSCs (as fructans) may also be involved in many stress-tolerant mechanisms[1] (Valluru *et al.*, 2008; Valluru and Van den Ende, 2008; Van den Ende and Valluru, 2009) and acts as osmolytes enhancing water retention under adverse environmental conditions (Kawakami *et al.*, 2008).

[1]Stress-tolerant mechanisms include protecting membranes and maintaining osmosis under chilling, drought stress conditions, acting as scavengers of reactive oxygen species in chilling-induced oxidative stress.

Stem WSCs accumulation is influenced by environmental factors (Ruuska *et al.*, 2006; Ehdaie *et al.*, 2006a,b). However, a significant genotypic variation in stem WSCs has been observed in wheat, which is consistent across environments with large broad sense heritability (H = 0.7-0.9) (Ruuska *et al.*, 2006). Earlier studies have emphasized using WSCs as a selection criterion in breeding programmes (Foulkes *et al.*, 2002; van Herwaarden *et al.*, 2003; Shearman *et al.*, 2005; Ruuska *et al.*, 2006). Indeed, an indirect selection for high stem WSCs occurred during the development of drought-tolerant wheat varieties in Australia and UK (Foulkes *et al.*, 2002; van Herwaarden *et al.*, 2003). A recent study found the quantitative trait loci (QTL) located on chromosomes 6D and 7A have shown a large effect on WSCs in wheat (Huynh *et al.*, 2008). Moreover, numerous candidate genes of carbohydrate synthetic pathway collocated with QTL. In barley grown in a drought environment, an additive QTL for leaf WSCs shared some chromosome zones with QTL for plant water status and/or osmotic adjustment by forming clusters of QTL (Teulat *et al.* 2001). Galiba *et al.* (1997) located a gene responsible for stem WSCs during cold acclimation on chromosome 5A of wheat. This suggests

that the selection for greater WSCs may have dual strategic effects; improving grain yields as well as plant adaptation across a range of environmental stresses (Foulkes *et al.*, 2007; Ehdaie *et al.*, 2006a,b; 2008). These studies strongly propose that WSC is a *trait of interest,* which can improve plant performance in both agronomical and physiological viewpoint (Foulkes *et al.*, 2007). However, despite the greater recognition as a quantitative trait, relatively little information exists on the morpho-physiological traits regulating WSC accumulation in wheat, particularly under different N levels.

The assimilatory processes of nitrogen (N) and carbon (C) are interrelated. It is not surprising; therefore, that the disruption in the supply of either of the resources can impose marked restrictions on the assimilation of the other (Lattanzi *et al.*, 2005; Bertheloot *et al.*, 2008). During nitrate assimilation, the carbohydrate synthesis is decreased as more carbon is converted to phosphoenolpyruvate through glycolysis and enters organic acid metabolism (Stitt *et al.*, 2002). Nitrate is known to affect several enzymes of C and N metabolism. Nitrate induced the expression of number of genes coding for the enzymes of organic acid synthesis (Rufty *et al.*, 1988), and the expression of the regulatory subunit of ADP-glucose pyrophosphorylase and increased starch and sucrose synthesis (Ruuska *et al.*, 2008). Consistently, nitrate stress is a negative signal, rapidly inducing fructan accumulation together with an induction of fructosyltransferases (FTs, enzymes responsible for fructan synthesis) (Wang & Tillberg, 1996; Morcuende *et al.*, 2004; Shiomi *et al.*, 2006; Ruuska *et al.*, 2008). Indeed, N deficiency strongly stimulated 6-SFT transcripts in barley leaves (Wang *et al.*, 2000) and 1-SST activity in chicory (Van den Ende *et al.*, 1999)[1]. The enhanced FTs may regulate fructan accumulation either directly or via downstream effects on the plant growth or metabolism under N stress (Wang *et al.*, 2000). Further, N deficiency elevated the transcripts for a number of protein or sugar kinases associated with the regulation of C storage in wheat (Ruuska *et al.*, 2008).

[1]In addition, N stress also affected fructan degrading enzyme, fructan exohydrolases (1-FEH)

Carbohydrates, in turn, play a regulatory role in nitrogen metabolism. For example, sucrose and glucose enhances the activation state of nitrate reductase (Morcuende *et al.*, 1998) and ferredoxin-glutamate synthase (Ruuska *et al.*, 2008). Further, sugars or sugar-phosphates act as signals regulating protein kinases and phosphates involved in the modulation of nitrate reductase activity (Ruuska *et al.*, 2008). Moreover, low sugar concentrations suppress nitrate reductase gene (*NIA*)

expression, overriding signals derived from nitrate and nitrate metabolism (Klein *et al.*, 2000). When sugars are low, they also affect amino acid synthesis at sites downstream from nitrate assimilation (Matt *et al.*, 1998). In addition, a depression of carbohydrate metabolism has been observed during active fructan synthesis, accompanied by low levels of phosphorylated intermediates (Pérez *et al.*, 2001).

As N negatively modulates FTs and fructans, the aim of this study was to identify several morphological and physiological traits associated with WSCs accumulation in wheat under different N levels. We developed a sensitivity index for genotypes ranking according to their capacity for WSCs accumulation. To this end, we then quantified trait correlations affecting WSCs by estimating the degree of trait sensitiveness employing selection analysis on the data derived under different N levels. Finally, the data were fitted into a predefined path model to define the strong determinants for WSCs accumulation under different N levels.

3.3 Materials and methods

3.3.1 Plant material

Eight winter wheat (*Triticum aestivum* L.) genotypes, Tommi, Biscay, Cetus, Contra (modern), Heines VII, Jubilar (old), *T. monococcum* and *T. dicoccum* (primitive species) were evaluated. The genotypes have different phenotypic characteristics differing in growth parameters, yield attributes and protein contents (Ward *et al.*, 2008; Weber *et al.*, 2008). Old genotypes[1] differ in tillering capacity and maturities (Bohn *et al.*, 1999). Primitive species differ in plant sizes; *T. monococcum* (diploid species) has long internodes with narrow leaves, whereas *T. dicoccum* (tetraploid species) has short internodes with wider leaf blades. Also, they differ in soluble sugars (Erdei *et al.*, 2002), protein contents (Hidalgo and Brandolini, 2008), soil nitrogen uptake (Trčková *et al.*, 2005), and growth and development (Biswas *et al.*, 2008).

[1]Jubilar has short culm and medium tillering capacity; while Heines VII is short culmed and medium maturity genotype.

3.3.2 Field experimental details

Three experiments were carried out in the field sites of Ihinger Hof Research Station (IHO, Exp. 1; 2007-08) and Oberer Lindenhof Research Station (OLH,

Exp. 2; 2007-08), and third under greenhouse conditions (Exp. 3, 2007) at the Institute of Crop Production and Grassland Research, University of Hohenheim, Germany. The field sites (IHO: 48° 44' N, 8° 56' E; OLH: 48° 28' N, 9° 18' E) have a temperate climate with wet, cold winters and warm summers, but differ in altitudes (450 and 700 m asl at IHO and OLH, respectively). The daily mean temperatures during the crop growing period were 7.7 and 7.2 °C and total precipitation was 698 and 854 mm at IHO and OLH, respectively. The soils at both stations are loamy.

Sowings were done on 14 and 16 October 2007 at IHO and OLH, respectively. The experiments were laid out in a randomized complete block design with three replications. Each plot consisted of 40 rows (6 x 4 m^2) and 20 rows (12 x 2 m^2) with an inter row spacing of 10 cm at IHO and OLH, respectively. N was applied at three levels comprising of 0 (low), 100 (medium) and 200 (high) kg ha^{-1} as ammonium nitrate in three splits; 30 and 60 kg N ha^{-1} at beginning of the vegetative stage (Zadoks scale 21); 40 and 80 kg N ha^{-1} at start of stem elongation (Zadoks 31); and 30 and 60 kg N ha^{-1} at end of the stem elongation (Zadoks 39) at IHO and OLH, respectively. Fungicied and herbicides were used to lower incidence of fungal diseases and weeds, respectively. When plants matured, harvesting was completed on 14 August 2008 at both stations using a Hege 180 (Hege, Germany) plot combine harvester.

3.3.3 Greenhouse experiment

Imbibed seeds of all genotypes were treated with 4 °C (42 d) to fulfil vernalization[2] requirement (Purvis and Gregory, 1952). Seedlings of about 2-3 cm were transplanted (1 April 2008) in square-shaped plastic pots (15 x 15 x 15 cm, 3 per pot), each filled with 4 kg mixture of sterile soil, organic matter, and sand (4:2:1 v/v), and placed in the glasshouse. On the day of complete emergence of first leaf (after 5-7 d), all pots were moved to field conditions. The experimental design used was a completely randomized block with five replications. Each pot represents a single block that contains three plants, and 180 blocks for all species were maintained. Soil moisture was maintained close to field capacity by supplying water everyday. N solution was applied (0, 100 and 200 kg N ha^{-1}) at different growth stages (see field experimental details). Nutrient solution containing P and K (10 mM) was applied regularly once in a week (100

[2]Vernalization is the quantitative requirement of low temperature (4 0C), which provide the competence to flower in temperate species.

ml pot^{-1}). Weeding was carried out regularly. The average relative humidity, solar radiation, average temperature and rainfall were 72%, 1874 J cm^{-2}, 15 °C, and 650 mm, respectively, representing optimal conditions during the crop growing period. Climatic data was obtained from Institute for Physics and Meteorology, University of Hohenheim.

3.3.4 Vegetative and reproductive trait measurements

Observations were recorded at two stages, namely at anthesis stage (Zadoks scale 65, Zadoks *et al.*, 1974), and 2 weeks after anthesis (Zadoks 69). In each experiment, 180 plants were sampled at random at each stage. All above-ground plant material was separated into leaf, stem, and root. Vegetative parameters such as plant height (excluding spikelet), leaf number, tiller number, flag-leaf length, width and flag-leaf area, peduncle length were measured and flowering time was determined. Dry weights of each plant sample were recorded after oven-drying at 65 °C for 72 h. Based on the dry mass, growth analysis for net assimilation rate (NAR) as the increase in plant mass per unit leaf area and time were calculated. Additionally, specific leaf area (SLA) as the ratio of fully expanded leaf lamina area to its dry mass, and leaf, stem and root mass ratio (LMR, SMR and RMR) as the ratio of leaf, stem and root dry weight to total plant dry weight was calculated, respectively. Crop growth rate (CGR) was calculated as the increase in plant mass per unit time. Based on these parameters, relative growth rate (RGR) was calculated (NAR* SLA* LMR) (Atkin *et al.* 2006). Further, we calculated leaf area index (LAI) as the ratio of total leaf area to ground area and quantified per cent allocation to roots, stems, leaves and ears for each individual as the ratio of the dry weight of each structure to total dry weight. Root: shoot ratio was calculated as the ratio of total dry weight of roots to that of leaves.

Before each destructive sampling, chlorophyll content was measured on the youngest leaf with portable SPAD chlorophyll meter (SPAD-502, MINOLTA, Japan). Net radiation was measured near each plot using digital luxmeter (MAVOLUX 5032C/b USB; GOSSEN GmbH, Germany) to calculate light extinction coefficient (*K*) based on the theory of Monsi and Saeki (1953). Radiation use efficiency[1] (RUE, g MJ^{-1}) was calculated using *K*, LAI, CGR and

[1]RUE is the amount of photosynthetically active radiation use to produce a unit biomass under non-stressed conditions.

net radiation using the equation, RUE = CGR / S_0 (1 — exp (K * LAI)) (Takai *et al.*, 2006).

3.3.5 Chlorophyll fluorescence and CO_2 assimilation

Measurements for chlorophyll fluorescence were made on the youngest leaf at 25 °C at a photosynthetic photon flux density (PPFD) of 1200 μmol m^{-2} s^{-1} to ensure light saturation. Maximal fluorescence (Fm) and dark fluorescence yield (Fo) were determined under shade, whereas steady-state fluorescence (F) and maximal fluorescence in the light-adapted state (Fm') produced by a 0.5-s saturating flash (PPFD of 10,000 μmol m^{-2} s^{-1}), were determined during morning times using a portable modulated fluorimeter (PAM-2000, Walz, Effeltrich, Germany). The Φ_{PSII} was determined from the equation (Genty *et al.* 1989) as $\Phi_{PSII} = (F'_m - F) / F'_m = \Delta F / F'_m$. The ΦCO_2 was determined using a model developed for wheat, $\Phi_{CO2} = ((F'_m - F_S / F'_m) - 0.0085) / 7.94$ (Keiller and Walker, 1990). Based on the $\Phi PSII$, the true rate of O_2 evolution was calculated using the model, $JO_2 = \Phi PSII * I_L / 10$ (Edwards and Baker, 1993) where I_L is average fractional absorbance of light by mature, healthy leaves (assumed 0.84; Demmig and Bjorkman, 1987). The photochemical (qP)[1] and non-photochemical quenching (NPQ)[2] parameters were measured. The partitioning of non-cyclic electron flow between CO_2 assimilation and photorespiration was studied by measuring responses of CO_2 assimilation and PSII quantum efficiency to intracellular CO_2 concentrations (C_i) at normal O_2 (21%) conditions. Non-cyclic electron flow[3] (J_L) was calculated as $J_L = \Phi_{CO2}$ x PPFD x 4 (Cheng *et al.*, 2001).

[1]Photochemical quenching refers to the ability of the transfer of electrons from photosystem II (PSII) to other alternate sinks to prevent photoinactivation of PSII.

[2]Non-photochemical quenching refers the thermal dissipation of excess excitation energy in the photosystem II antenna.

[3]Non-cyclic electron refers the first stage of photosystem in which the released electrons do not come back to the source and used to producing ATP and NADH, which enter calvin-cycle to produce carbohydrates.

3.3.6 WSCs and nitrogen analyses

Dried samples of leaf, stem and root and were analysed for total N (Dumas, 1962) using a Heraeus macro-N analyzer (Hanau, Germany). Water-soluble carbohydrates were analysed only in leaf and stem samples by a modified HPLC method of Turner et al., (2006). Water-soluble carbohydrates (WSCs) were analyzed by high-performance liquid chromatography (HPLC). For extraction of WSCs, ground plant material was weighed in 2 ml screw-capped plastic tubes and 1.5 ml of hot demonized water was added. Tubes were sealed, vigorously shaken in a thermomix for 10 min at 85 °C and 30 min at 25 °C, and then

centrifuged at 20 °C for 10 min. The aqueous extract was filtered with 0.45 µm syringe filter into HPLC vials. Sugars were separated and quantified by HPLC on a 300 x 7.8 mm column of Phenomenex Zezex RCM-Monosaccharide Ca^{+} (BISCHOFF Chromatography GmbH, Leonberg, Germany) at 85 °C with degassed water as a mobile phase, at 0.6 ml min^{-1} for separation of fructan and sucrose. Samples were analyzed using a RI-8100 refractive index (RI) monitor, 2200 series pump, AS-728 auto-sampler and high-resolution liquid chromatography instrument interface (BISCHOFF Chromatography GmbH, Leonberg, Germany). Aqueous solutions (range 100–5000 ppm) of commercially available carbohydrates (inulin, sucrose, glucose and fructose, Sigma, Germany) were used as standards.

3.3.7 Statistical analyses

Trait means for each species within each N level were calculated. For the analyses, non-transformed data were mostly used, unless data were not normally distributed. Pearson correlations among all traits (independent factors) and WSCs (dependant variable) were estimated separately because of strong collinearity[1] between traits. To examine variation in the phenotypic architecture within each nitrogen level, we calculated phenotypic variance-covariance matrices (*P*-matrices) for the traits adjusted to within-treatment standard deviation, with the null-hypothesis that the matrices are unrelated.

To examine which traits are the strongest determinants of WSCs, total and direct selection on all traits was calculated for each nitrogen level separately, using the data on individuals of all eight species. We used the total WSCs as an estimate of fitness. To estimate relative fitness within each N level, the genotypic mean for WSCs was divided by the within-treatment grand mean (Rausher, 1992). Standardized selection differentials[2] (S) were calculated on the traits adjusted to within nitrogen level standard deviation units as the covariance of relative fitness and the particular trait. Significance of selection differentials was assessed via Pearson correlations. Selection gradients were estimated for all traits separately using multivariate regression analysis (Lande and Arnold, 1983). Directional selection gradients[3] (β) were calculated as a partial linear regression coefficient of relative fitness on the standardized traits.

[1]Collinearity is a statistical phenomenon in which two or more predictor variables in a multiple regression analysis are highly correlated.

[2]Selection differential is the covariance between a trait mean and its relative fitness. It describes total selection or the difference between the mean of a population and the mean of individuals selected to be parents of next generation.

[3]Selection gradient is the partial regression coefficient between a trait and relative fitness. It describes direct selection.

Path analysis was used to visualize the complex relationship between multiple traits and individual fitness in three N levels. We fitted a model with three hierarchical levels (Fig. 1). In this model, the vegetative traits (vegetative mass, flag-leaf width, root/shoot ratio) directly influenced physiological traits (chlorophyll per unit leaf area, leaf nitrogen, net assimilation rate, radiation use efficiency, and quantum yield of CO_2 assimilation), and latter, in turn, directly affect fitness estimated by total WSCs. All possible path correlations between all traits were modeled. The path analysis was performed for each N level separately using structural equation modeling (LISREL; Joreskog and Sorbom, 1988).

To estimate genotypic sensitivity index (*GSI*), WSCs means for each species within each N environment were calculated as an estimate of genotype means. We assessed the relative difference between the genotypes for WSCs accumulation. For this, WSCs values were first log transformed and then standardized to distributions with mean zero and standard deviation one (Sokal and Rohlf, 1995), $X_{carbohydrates} = X - \overline{X} / s$, where X is the replicated value; $\overline{X}$ is the grand mean within each N treatment; s is standard deviation. *GSI* values were calculated for each species separately under each N level. Broad sense heritabilities (h^2) were computed from the estimates of genetic (σ_g^2) and residual variances (σ_e^2) derived from the expected mean squares as $h_g^2 = \left[\sigma_g^2 / \left(\sigma_g^2 + \sigma_e^2 / k\right)\right]$, where k is the number of replications (Yang *et al.*, 2007).

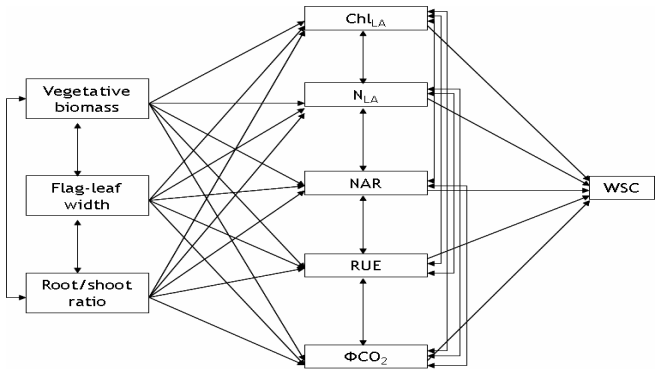

Figure 1 Path model used for estimating selection on water-soluble carbohydrates (WSCs) in wheat.

3.4 Results

3.4.1 Variation for total WSC and positive correlation between WSC concentration and grain number or grain weight

There was a significant variation in total WSCs among wheat genotypes. The WSC concentration in the highest WSC genotype was about 27% higher than that in the lowest genotype (Fig. 2). The differences in WSC concentrations among these genotypes were similar in all experiments, suggesting that the variation was largely genotypic. Fructan was the major component that contributed to the genotypic variation in WSC concentration (r^2 = 0.80***, Fig. 3A). In addition, sucrose positively contributed to the WSC, but was not statistically significant (r^2 = 0.58, Fig. 3B).

However, the combined levels of fructan and sucrose were significantly correlated with the WSC concentrations (r^2 = 0.81**, Fig. 3C). Glucose and fructose levels were inversely, but not statistically significant, associated with WSC concentrations (r^2 = 0.41 and 0.30, respectively, Fig. 3D). To determine the relationships between WSCs and grain yield or weight, the WSC levels of individual genotype replicates were plotted with grain yield or weight. Significantly strong correlations between WSCs and grain yield (r^2 = 0.94**, Fig. 3E) and grain weight (r^2 = 0.88**, Fig. 3F) were observed among eight genotypes.

3.4.2 Traits regulating total WSCs

All traits evaluated exhibited significant correlations with total WSCs contents (Table 1). A few of them, however, were consistently expressed across N levels. Among 35 traits, 28 traits were significantly, positively or negatively, correlated with total WSCs contents in all N levels, and 22 of them were consistent across N levels. Among all traits, root: shoot ratio (RS_R, r^2 = –0.92, $P < 0.001$), stem nitrogen (S_N, r^2 = –0.99, $P < 0.001$), leaf nitrogen (L_N, r^2 = –0.48, $P < 0.01$) and nitrogen per unit leaf area (N_{LA}, r^2 = –0.43, $P < 0.001$) were negatively correlated with total WSCs contents in all N levels. This suggests that plant N is a negative signal for total WSCs contents; indeed, a unit increase in leaf N resulted in 28% decrease in total WSCs contents. V_{MASS} was negatively, but

marginally significant, associated with total WSCs contents in low and high N, but not in medium N level where it was marginally positive.

We selected three vegetative and five physiological traits (Table 2) for further analysis with the assumption that the over-expression of any of these traits for theoretical WSC gains is possible because of the mechanistic and genetic linkages among these traits as well as with total WSCs contents, although not yet fully established, might be linked.

The total WSCs contents were higher in medium N level, and were 17 and 25% higher than in low and high N level, respectively. In contrast, higher V_{MASS} was found in high N level, and was 62 and 32% higher than in low and medium N level, respectively. This resulted in a negative correlation between total WSCs contents and V_{MASS} under low (r^2 = -0.62, P < 0.001) and high (r^2 = -0.71, P < 0.001) N levels, but marginally positive correlation under medium (r^2 = -0.46, P < 0.041) N level. All these traits were highly heritable (Table 2).

Table 1 Pearson correlation (*r*) of several morpho-physiological traits with total water-soluble carbohydrates (WSC) under three N levels in eight wheat genotypes.

Trait	Description	N_0		N_{100}		N_{200}	
		r	*p*	*r*	*p*	*r*	*P*
H_P	Plant height	0.197	0.356	0.200	0.348	0.181	**0.002**
LN	Leaf number	0.415	**0.009**	0.018	0.934	0.003	0.986
T_N	Tillers number	0.733	**0.001**	0.783	**0.001**	0.825	**0.001**
FL_L	Flag-leaf length	0.021	0.920	0.053	0.805	0.196	0.358
FL_W	Flag-leaf width	0.913	**0.003**	0.179	0.402	0.118	**0.003**
FL_A	Flag-leaf area	0.073	0.733	0.108	**0.004**	0.185	0.387
P_L	Peduncle length	0.524	**0.004**	0.335	**0.001**	0.299	**0.006**
FT	Flowering time	0.693	**0.001**	0.627	**0.001**	0.722	**0.001**
SLA	Specific leaf area	0.043	0.839	0.049	0.817	0.041	0.846
LMR	Leaf mass ratio	0.848	**0.001**	0.625	**0.001**	0.676	**0.001**
SMR	Stem mass ratio	0.910	**0.001**	0.883	**0.001**	0.736	**0.001**
RMR	Root mass ratio	0.892	**0.001**	0.815	**0.001**	0.690	**0.001**
CGR	Crop growth rate	0.071	0.738	0.036	0.867	0.197	0.356
NAR	Net assimilation rate	0.613	**0.008**	0.088	**0.002**	0.234	**0.002**
RGR	Relative growth rate	0.218	0.306	0.300	0.154	0.440	**0.031**
LAI	Leaf area index	0.549	**0.005**	0.613	**0.001**	0.172	0.421
RS_R	Root/shoot ratio	-0.929	**0.001**	-0.878	**0.001**	-0.803	**0.001**
V_{Mass}	Total vegetative dry mass	-0.859	**0.004**	0.028	**0.001**	-0.023	**0.007**
L_N	Leaf nitrogen	-0.488	**0.015**	-0.813	**0.001**	-0.553	**0.005**
S_N	Stem nitrogen	-0.993	**0.001**	0.971	**0.001**	0.888	**0.001**
N_{LA}	Nitrogen per unit leaf area	-0.434	**0.001**	0.424	**0.039**	0.738	**0.001**
C_{LA}	Carbon per unit leaf area	0.101	0.638	0.025	0.905	0.228	0.284
Chl	Chlorophyll content	0.033	0.878	0.100	0.641	0.106	0.623
Chl_{LA}	Chlorophyll per unit leaf area	0.793	**0.004**	0.042	0.845	0.265	**0.005**
K	Light extinsion coefficient	0.996	**0.001**	-0.989	**0.001**	-0.968	**0.001**
RUE	Radiation use efficiency	0.821	**0.004**	0.239	**0.002**	0.128	**0.002**
ΦPSII	Quantum efficiency of photosystem II	0.072	0.738	0.205	0.336	0.398	**0.054**
ΦCO_2	Quantum yield of CO_2 assimilation	0.987	**0.001**	0.990	**0.001**	0.973	**0.001**
ΦCO_2/ ΦPSII	ΦCO_2/ ΦPSII ratio	0.975	**0.001**	0.964	**0.001**	0.948	**0.001**
JO_2	Rate of O_2 evolution	0.495	**0.013**	0.577	**0.003**	0.676	**0.001**
qP	Photochemical quenching	-0.392	0.057	-0.457	0.024	-0.561	0.004
NPQ	Non-photochemical quenching	-0.251	0.238	-0.269	0.203	-0.334	0.111
J_L	Non cyclic electron flow	0.062	0.772	0.032	0.882	0.080	0.710
C_L	Cellulose	0.876	**0.001**	0.782	**0.001**	0.947	**0.001**
H_C	Hemicellulose	0.881	**0.001**	0.838	**0.001**	0.895	**0.001**

Table 2 *F*-statistics for one-way ANOVA and mean (±SE) trait values and heritabilities for different morpho-physiological traits in three nitrogen levels across eight wheat genotypes.

Trait	*F* (d.f. 2, *n*=44)	Treatments			
		N_0	N_{100}	N_{200}	h^{2*}
V_{MASS}	108.91***	2.49 ± 0.05	4.59 ± 0.05	6.75 ± 0.15	0.99/0.99/0.99
FL_W	49.43***	1.35 ± 0.03	1.51 ± 0.01	1.70 ± 0.02	0.89/0.95/0.99
RS_R	205.70***	0.711 ± 0.01	0.56 ± 0.01	0.41 ± 0.01	0.94/0.86/0.98
Chl_{LA}	71.79***	15.77 ± 0.74	27.42 ± 0.46	43.78 ± 2.15	0.83/0.98/0.92
N_{LA}	107.39***	1.12 ± 0.02	1.30 ± 0.01	1.50 ± 0.02	0.96/0.94/0.99
NAR	39.47***	6.90 ± 0.23	9.69 ± 0.28	12.74 ± 0.52	0.55/0.84/0.43
RUE	29.18***	9.51 ± 0.82	16.05 ± 0.45	22.55 ± 1.37	0.64/0.86/0.91
ΦCO_2	39.63*	0.04 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.91/0.81/0.49
WSC	214.80***	203.30 ± 2.04	247.81 ± 4.40	184.90 ± 7.42	0.84/0.94/0.94

*For heritabilities, the values at the left, middle and right indicate for N_0, N_{100} and N_{200} treatments, respectively. $V_{MASS,}$ vegetative dry biomass; $FL_{W,}$ flag-leaf width; RS_R, root/shoot ratio; $Chl_{LA,}$ chlorophyll per unit leaf area; N_{LA}, nitrogen per unit leaf area; NAR, net assimilation rate; RUE, radiation use efficiency; $\Phi CO_{2,}$ quantum yield of CO_2 assimilation; WSC, water-soluble carbohydrates.

Table 3 Standardized selection differentials and gradients of several morpho-physiological traits under three N levels across eight wheat genotypes.

Traits	Selection differential			Selection gradients		
	N_0	N_{100}	N_{200}	N_0	N_{100}	N_{200}
V_{MASS}	**0.031*****	**-0.186*****	**-0.701*****	**0.079** (± 0.04)*	**-0.069** (± 3.36)***	**-0.089** (± 0.02)***
FL_W	**0.020*****	**0.010****	**-0.006****	**0.156** (± 0.15)**	0.943 (± 2.63)	**-0.545** (± 0.20)*
RS_R	**-0.001***	**-0.002***	**0.002****	**-0.355** (± 0.42)**	-0.152 (± 2.74)	0.049 (± 0.33)
Chl_{LA}	0.915	0.191	0.913	0.277 (± 0.01)	0.246 (± 0.65)	-0.052 (± 0.02)
N_{LA}	**-0.005*****	**-0.001*****	**-0.006****	**-0.916** (± 0.02)*	**-0.330** (± 2.84)*	**-0.998** (± 0.02)***
NAR	0.139	0.050	0.765	0.060 (± 0.01)	0.075 (± 2.96)	-0.298 (± 0.07)
RUE	**0.646*****	**0.340*****	**0.535*****	**0.418** (± 0.01)**	**0.220** (± 1.84)***	**0.016** (± 0.03)***
ΦCO_2	0.001	0.001	0.001	0.308 (± 0.01)	0.427 (± 3.10)	-0.033 (± 0.01)

$V_{MASS,}$ vegetative dry biomass; $FL_{W,}$ flag-leaf width; RS_R, root/shoot ratio; $Chl_{LA,}$ chlorophyll per unit leaf area; N_{LA}, nitrogen per unit leaf area; NAR, net assimilation rate; RUE, radiation use efficiency; $\Phi CO_{2,}$ quantum yield of CO2 assimilation; WSC, water-soluble carbohydrates.

Table 4 Comparative analysis of selected morpho-physiological markers between high and low WSCs species and modern, old and primitive species across N levels. Values are means ± SE of five species (for high WSCs species), three species (for low WSCs species) derived from two field and one glasshouse experiments.

Groups	WSCs (mg g^{-1})	V_{MASS} (g)	RUE (g MJ^{-1})	JO_2	ΦCO_2	RS_R	N_{LA}
High WSCs species	209.62 ± 9.50	3.59 ± 0.179	14.95 ± 0.643	0.03 ± 0.002	0.08 ± 0.004	0.54 ± 0.018	1.31 ± 0.024
Low WSCs species	184.53 ± 7.36	4.45 ± 0.139	13.86 ± 0.498	0.05 ± 0.012	0.05 ± 0.003	0.61 ± 0.013	1.39 ± 0.019
P value	***	***	**	*	*	***	***
Modern species	190.00 ± 8.440	4.92 ± 0.151	15.59 ± 0.545	0.05 ± 0.001	0.07 ± 0.015	0.63 ± 0.015	1.43 ± 0.021
Old species	198.27 ± 11.93	3.69 ± 0.021	13.46 ± 0.771	0.04 ± 0.013	0.06 ± 0.213	0.56 ± 0 021	1.41 ± 0.029
Primitive species	214.57 ± 11.91	4.23 ± 0.021	13.53 ± 0.742	0.03 ± 0.002	0.06 ± 0.211	0.53 ± 0 021	1.31 ± 0.029
P value	**	***	***	**	*	***	**

***P <0.001, **P <0.01, *P <0.05.

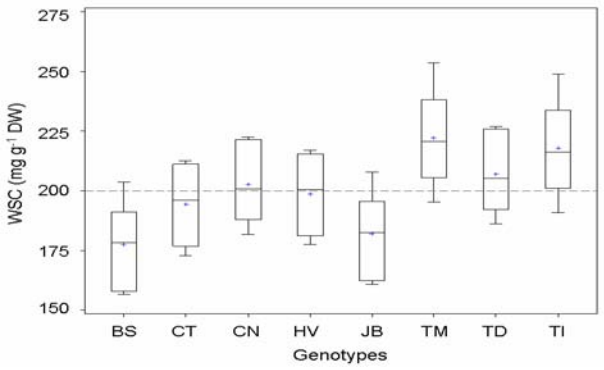

Figure 2 Total WSCs in eight wheat genotypes at anthesis. Values are means of two field and one glasshouse replicates. Horizontal dashed line represents mean of all genotypes. BS, Biscay; CT, Cetus; CN, Contra; TI, Tommi; JB, Jubilar; HV, Heines VII; TM, *Triticum monococcum*; TD, *T. dicoccum*; DW, dry weight.

3.4.3 Selection differential and gradients

To assess further the degree of trait adaptiveness and determinacy in regulating total WSCs contents, phenotypic selection analysis was adopted. This revealed that one morphological trait, V_{MASS} and two physiological traits, N_{LA} and RUE showed significant selection differentials in all N levels, suggesting that these traits were strongly favoured in all N levels (Table 3). In the presence of low and

medium N level, high V_{MASS} was favoured, as indicated by a strong positive selection differential; whereas, low V_{MASS} was favoured in high N levels, as indicated by strong negative selection differentials. The selection gradients also indicated that high V_{MASS} in low and medium N and low V_{MASS} in high N level were favoured.

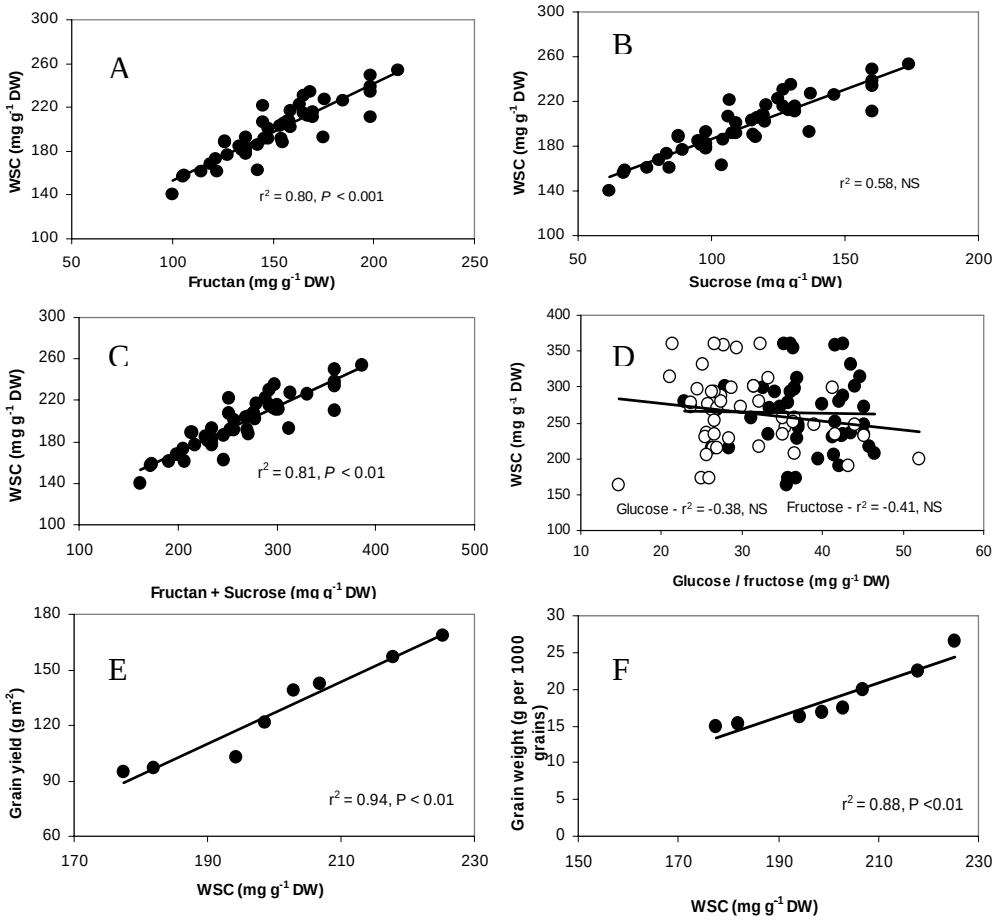

Figure 3 Relationships between WSCs, fructan (A), sucrose (B), fructan+sucrose (C), glucose and fructose (D), grain yield (E) and grain weight (F) in eight wheat genotypes at anthesis differing in WSCs. Each point represents a single genotype replicate. DW, dry weight; NS, not significant.

In all N levels, lower N_{LA} was favoured; however, a strong selection gradient in high N level indicated that, for plants that attain higher V_{MASS}, selection favours lower N_{LA} that increase total WSCs contents (Table 3). In contrast, higher RUE was favoured in all N levels, as indicated by strong positive selection differential, which is also supported by positive selection gradients. The RS_R, which represents the pulses of resource allocations within

the plant, showed that the lower RS_R was favoured in low and medium N level; whereas higher RS_R was favoured in high N

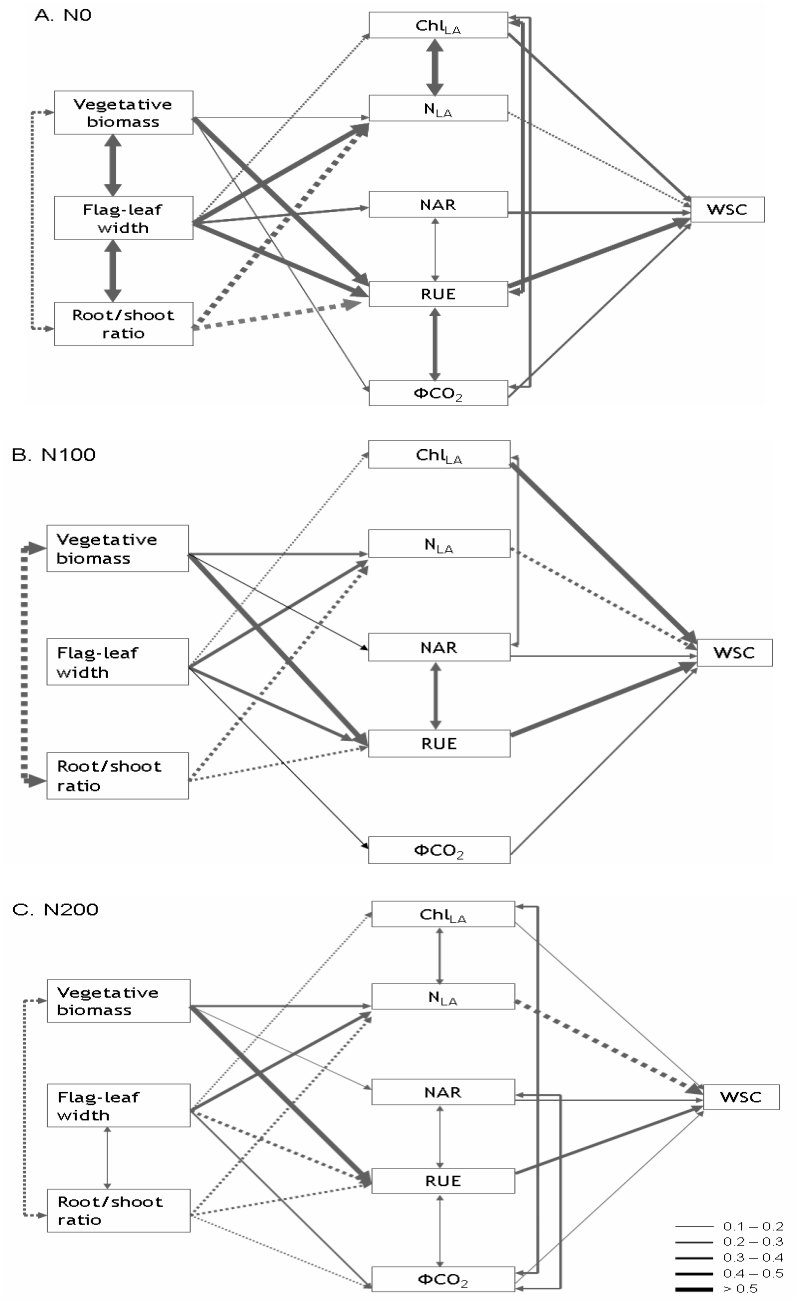

Figure 4 Path analysis of selection on WSCs under three nitrogen levels. Solid and dashed lines denote positive and negative relationship, respectively. Chl_{LA}, chlorophyll per unit leaf area; NAR, net assimilation rate; RUE, radiation use efficiency; ΦCO_2, quantum yield of CO_2 assimilation.

level, also consistent with the selection gradients. The selection differentials of other traits varied across N levels in both strength and sign. As trait correlations with total WSCs contents (selection differentials) for some traits were different from the corresponding partial regression coefficients for those traits (selection gradients), the effects of these traits on total WSCs contents seemed to be partly indirect and mediated by other traits.

3.4.4 Path analysis for maximizing WSCs

The visual inspection of path coefficients revealed how vegetative traits affected the fitness (WSCs contents) through the intermediary physiological traits, and through the correlations with other vegetative traits. Path coefficients of physiological traits on fitness were similar in all N levels, with strong positive effects of RUE ($r^2 = 0.72$, $P < 0.001$), NAR ($r^2 = 0.4$, $P < 0.001$) and Chl_{LA} ($r^2 = 0.51$, $P < 0.001$), the moderate positive effect of ΦCO_2 ($r^2 = 0.29$, $P < 0.001$) and a strong negative effect of N_{LA} ($r^2 = -0.63$, $P < 0.001$) (Fig. 4). Some important paths from the vegetative traits to intermediary physiological traits, however, seemed to be consistent and unaffected by N levels: (a) a strong positive effect of V_{MASS} on RUE; (b) a positive effect of FL_W on N_{LA} under low and high N, but negative effect on N_{LA} under medium N level; (c) a negative effect of RS_R on N_{LA} and RUE; and (d) a negative correlation between V_{MASS} and RS_R. These N-specific correlations, positive or negative, between these traits seem to regulate the total WSCs contents under different N levels.

3.4.5 Genotypic sensitivity index (GSI)

Among all N levels, the genotypes under optimum N showed, on average, higher *GSI* value whereas high N has lower *GSI* values (Fig. 5). The *GSI* values for the high N group extended to lower values and differed significantly with the low and optimum N group. Across N levels and genotypes, *T. monococcum* (TM) had a higher *GSI*, while Tommi (TI) followed closely. Moreover, for all species that showed higher WSC concentrations, the *GSI* values were positive, and for all species having lower WSCs levels, *GSI* values were negative (Fig. 5). A comparative analysis of selected traits between five

highest and three lowest WSC genotypes as well as between four modern, two old and two primitive species revealed that high and low WSC genotypes differed in resource allocations and N_{LA}. The modern, old and primitive species differed largely in RUE and resource allocations (Table 4).

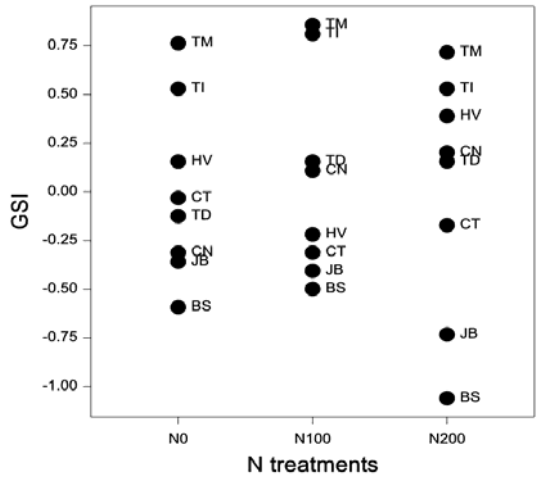

Figure 5 Genotype sensitivity index (*GSI*, standardized log values of WSCs) under three levels of N for four modern (Biscay, BS; Cetus, CT; Contra, CN; Tommi, TI), two old (Jubilar, JB; Heines VII, HV) and two primitive species (*Triticum monococcum*, TM; *T. dicoccum*, TD).

3.5 Discussion

3.5.1 Ecological significance of WSC traits

Grain yield of cereals is a complex trait, which is fairly dependent on the WSCs accumulation and remobilization in plants. Correlations, univariate and multivariate regression and path analyses revealed a detailed view of selection on WSCs. In all N levels, the increase in total WSCs was primarily associated with V_{MASS}, FL_W, RS_R, RUE and N_{LA}. This is a generalized, yet genetically unexplored, pattern of relationships, particularly between FL_W, RS_R, RUE, N_{LA} and WSCs. The selection for these traits can set an upper limit to potential WSCs in wheat.

Higher V_{MASS} maximized WSCs under low and optimum N, but not under high N level. When N supply was high, the total selection favoured more V_{MASS},

which directly influenced RUE, and N_{LA} (Fig. 4). Under these conditions, despite the high investment of N_{LA}, which diminishes the return (WSCs gain) per unit investment, the relatively high magnitude of the effect of RUE might increase WSCs. When N supply was limiting, the total selection maximized two components of V_{MASS} investment: RUE and ΦCO_2, but not N_{LA}. The higher N_{LA} under N stress incurred a greater cost, but the lack of significant covariance with V_{MASS} favoured an increase in V_{MASS} to maximize total WSCs. Although the path diagrams showed nearly similar V_{MASS} investments under low and high N, the trait correlations and the lack of significant total selection on N_{LA} may suggest the existence of different N specific regulatory mechanisms under different N levels. When N was not limiting, plants may tend to retain all photosynthetically active organs (leaves) until their carbohydrate gain became nearly zero, thus maximizing and extending the N metabolism via more carbon conversion to phosphoenolpyruvate through glycolysis and enter organic acid metabolism (Stitt *et al.*, 2002; Morcuende *et al.*, 2004). On the other hand, when N was limiting, N deficit triggers the process of plant senescence and shedding of leaves may increase the WSCs gain of the whole plant. Plants increase the efficiency of N use at the whole plant level, even though their leaf C gain is still positive through increased leaf shedding (Yasumura *et al.*, 2007; Oikawa *et al.*, 2008).

Higher FL_W maximized WSCs under low N, but not in high N level. Leaf characteristics play a critical role in determining the plants fitness against abiotic stresses (Falster and Westoby, 2003; Hopkins *et al.*, 2008). In our study, the path models under different N levels revealed unique relations between leaf traits to maximize total WSCs (Fig. 4). The direct selection on FL_W under N stress negatively favoured Chl_{LA} and RUE, while N_{LA} was positively favoured. Higher FL_W, coupled with higher leaf thickness[1], is profitable when plants grow under N stress due to higher RUE and NAR (Shearman *et al.*, 2005; Izanloo *et al.*, 2008). In either ways, N stress-induced FL_W might display plastic correlations with N_{LA} and RUE to maximize the total WSCs. When N supply was not limiting, the selection maximized three components of FL_W: RUE, N_{LA} and ΦCO_2. This selection seemed interesting, because, FL_W equally favoured both RUE and N_{LA}. Apparently, plants under high N supply exhibited specific cost-effective morphological adaptations such as leaf rolling or erectness to improve RUE and maximizing total WSCs (Izanloo *et al.*, 2008; Hopkins *et al.*,

[1]Higher leaf thickness, an index of specific leaf area, represents more photosynthetic tissue per unit leaf area, and thus improves RUE.

2008). Greater leaf angles (more erectness) directly affect the flux of solar energy per unit leaf area and thus determine the maximum photosynthetic ability of a plant (Falster and Westoby, 2003). Further, the selection on adaptive leaf morphology (greater leaf angles) may decrease plant water loss (Kalapos and Csontos, 2003) and reduce biomechanical constraints[1] (Niklas, 1999). This might provide an adaptive explanation for the observed leaf trends under high N, and the selection on improved RUE might encompass the selection on N_{LA} and maximize total WSCs.

[1]Biomechanical constraints refer to the force required to hold up a leaf, which is more in lower leaf angles.

Lower RS_R maximized WSCs under low and optimum N but not under high N level. The direct selection on RS_R was observed only under N stress. Total selection on RS_R was positive under N stress, and negative under optimum N, displaying plastic allocations in aboveground vs. root biomass across N levels. A disproportionate allocation into root biomass is not profitable when nutrients are limiting (Volis *et al.*, 2004). This indicates that the high RS_R do not increase total WSCs. The increased translocation of carbohydrate pools to the root system probably is related, at least partly, to a decreased rate of leaf canopy development under N stress (Cruz *et al.*, 2003) resulting from the varied cell expansion and cell divisions (Dodd *et al.*, 2002) and altered conductance of the plants (Dodd *et al.*, 2002), possibly through interactions with a nitrate-cytokinin signaling pathway (Forde, 2002). The restricted leaf canopy development has two general effects: decrease in the rate of leaf initiation and expansion and decrease in the development of existing sink leaves. Both effects contribute to decreased utilization of carbohydrates in the shoots (Rufty *et al.*, 1988). However, as long as the restrictions imposed on the leaf development exceed the coincident decline in the photosynthetic rate of existing leaves, the system might initiate increased translocations of fixed carbohydrates to the root system. Nevertheless, RS_R, by favouring a negative relation with N_{LA} (Morcuende *et al.*, 2004) and a positive relation with ΦCO_2 (Ruuska *et al.*, 2008) maximized total WSCs under N stress.

3.5.2 Implications of whole plant carbohydrate and N interactions

N stores interact directly with the provision and utilization of carbohydrates (Lattanzi *et al.*, 2005; Bertheloot *et al.*, 2008). In suggesting a causal relationship between N and carbohydrate assimilation, two distinct trends are commonly

found: (a) a negative signalling between N and carbohydrate assimilation, consistent with our study and previous studies (van Herwaarden *et al.*, 1998, 2003; Morcuende *et al.*, 2004; Ruuska *et al.*, 2008; Rebetzke *et al.*, 2008); (b) the translocation of a large portion of carbohydrates to the root system under N stress (Rufty *et al.*, 1988; Dodd *et al.*, 2002; Cruz *et al.*, 2003).

When the plants have sufficient N, they synthesize more amino acids, develop more sinks, and improve sink capacity to use carbohydrates. The lower WSCs in high N-grown plants indicate that there was no limitation to use WSCs. Fixed N requires C skeletons for assimilation and further biosynthesis. Therefore, imported carbohydrates could be used to fuel biosynthesis in the developing leaves, possibly explaining why total WSC levels were lower in plants grown under high N than the plants grown under low N level (Ainsworth *et al.*, 2007). Previous studies, which calculated the C export by mass balance (Rogers *et al.*, 2004) suggest that the adjacent leaves (mostly developing leaves) are likely to be strong proximal sinks for carbohydrates, particularly during vegetative growth (Ainsworth *et al.*, 2007). Further, some of the WSCs produced during the day in high N-grown plants may be lost by respiration at night because an increase in carbohydrates correlated with increased respiration, and therefore represents respiratory substrates (Mizuno *et al.*, 2008).

On the other hand, in low N-grown plants, there was a large reduction in growth due to limited N. This resulted in a large accumulation of WSCs indicating that N is limiting for the synthesis of amino acids and sink development, therefore, reduced sink capacity to use WSCs. This suggests that (a) the growth was not limited by WSCs availability and, (b) the capacity for photosynthesis and carbohydrate synthesis is greater than sink capacity in low N-grown plants. Although long-distance signals related to higher carbohydrate status drives increased growth in developing leaves, it was not apparent by measurements of leaf area index under low N. The decreased utilization of WSCs might be due to the requirement of additional WSCs to serve an osmotic function (Kameli & Lösel, 1996). A limitation in the exogenous N supply could rapidly influence the endogenous N status and organic acids generated during nitrate reduction, both of which may be involved in turgor maintenance in leaf cells (Steingröver *et al.*, 1986). Alternatively, the leaves of low N-grown plants might have an increased capacity for carbohydrate

synthesis, possibly through an enhanced induction of metabolic enzymes. For example, N stress enhanced FTs activity (eg. 1-SST, 1-FFT), which rapidly increased fructan levels (Wang & Tillberg, 1996; Van den Ende *et al.*, 1999; Wang *et al.*, 2000; Ruuska *et al.*, 2008; Xue *et al.*, 2008). Indeed, a recent study found it was nitrate stress, and not a downstream metabolite in N assimilation, that negatively affected fructan synthesis in barley leaves (Morcuende *et al.*, 2004). Further, N stress seems to upregulate *agpS* transcript expression, which encodes the regulatory subunit of ADP-glucose pyrophosphorylase (AGPase; Scheible *et al.*, 1997) and allosteric activation of AGPase catalysis, the activities associated with starch metabolism (Ruuska *et al.*, 2008). Moreover, N stresses also upregulated the transcripts for a number of protein or sugar kinases associated with the regulation of carbohydrate storage (Ruuska *et al.*, 2008). The plants grown under N stress seem to have a decreased source: sink ratio due to partial shedding of the source leaves in ryegrass (Rogers *et al.*, 1998). Indeed, plants may enhance the efficiency of N use at whole plant level and trigger N retranslocation to maximize the whole-plant carbon gain (Oikawa *et al.*, 2008). Therefore, sink size and capacity plays a crucial role in the acclimation to N stress.

3.5.3 Genotype sensitivity to WSCs accumulation

Numerous studies have reported a vast genotypic variation for WSCs in wheat. Many factors, either environmental, morphological, physiological, enzymatic, biochemical mechanisms, or resource (N, water) use efficiency may contribute to WSCs variation (van Herwaarden *et al.*, 1998, 2003; Foulkes *et al.*, 2002, 2007; Morcuende *et al.*, 2004; Ehdaie *et al.*, 2006a,b, 2008; Ruuska *et al.*, 2006; Yang and Zhang, 2006; Xue *et al.*, 2008; Mizuno *et al.*, 2008; Ruuska *et al.*, 2008). However, the differences in the influx and/or efflux rate of carbon into the stem sucrose pools could primarily regulate WSCs accumulation. Further, a relative partition of carbon between WSCs and stem cell wall polysaccharides (cellulose and hemicellulose) is a crucial factor that could apparently influence the extent of WSCs storage in wheat (Antuono et al., 1998; Xue *et al.*, 2008).

We observed significant inter-species differences in *GSI* values in all N levels (Fig. 5). This suggests that all species have species-specific mechanisms regulating WSCs under different N levels. However, *T. monococcum,* Tommi and Heines VII consistently showed higher WSCs in all N levels[1]. Higher WSCs might be

[1]This suggests that the WSCs regulation in these genotypes was less sensitive to N availability.

partly due to the lower partition of carbohydrates to the roots, as also evident from lower RS_R (40%) (Table 4) (Cruz *et al.*, 2003). Further, these species have lower N_{LA} (6%). Alternatively, lower carbohydrate flux to stem cell wall polysaccharides (Antuono et al., 1998; Xue *et al.*, 2008), alterations in gas exchange, and chlorophyll fluorescence signals (Biswas *et al.*, 2008) may indeed contribute to higher WSCs. Interestingly, modern genotypes (except Tommi) have lower *GSI* values than primitive and old species. The causes of this discrepancy is unclear but may possibly be related to differential growths and physiological adaptations. For example, all modern genotypes showed higher RS_R (18%), which might have decreased total WSCs (Table 4) (Cruz *et al.*, 2003). Biswas *et al.* (2008) found higher RS_R and allocation coefficients in modern genotypes compared to primitive and wild wheat species.

Further, total biomass and total WSCs are two extreme ends, between which lie a range of possibilities that we expect to be related to strategies of growth, and physiology, in particular genetic architecture of the species (Biswas *et al.*, 2008). For example, the contribution of A, B, D genome to modern wheat (*T. aestivum* with AABBDD genome) was derived from *T. turgidum* spp. *durum* (AABB), *T. monococcum* (AA) and *Aegilops tauschii* (DD) (Biswas *et al.*, 2008). It has been reported that the inclusion of B genome into the A genome of *T. monococcum* reduced the negative effects of ozone stress on photosynthesis. In contrast, the addition of D genome into the AB genome of *T. turgidum* ssp. *durum* (the origin of *T. aestivum*) enhanced the negative effects of ozone stress (Biswas et al., 2008). These negative effects of D genome on the photosynthesis were also apparent on growth, dry matter accumulation, resource allocations and partitioning in modern wheat under abiotic stresses (Biswas *et al.*, 2008). The negative impacts of D genome on the photosynthetic performance were consistent across the literature (see Biswas et al., 2008 and the references therein). We, therefore, propose that the patterns of WSCs are important aspects of plants ecological strategies in locations subject to periodic N stress.

It is interesting to notice that high N decreased WSCs but increased total V_{MASS}, which are two positively correlated traits (Xue *et al.*, 2009). Further, genotypes with higher WSCs have lower V_{MASS} than low WSCs genotypes (Reynolds *et al.*, 2006). This suggests that N might act through a different, as yet unknown mechanism, breaking the general positive norm between V_{MASS}

and WSCs. This reinforces our earlier expectation that the positive association between WSCs and V_{MASS} may not be a general norm but the adapted physiological processes might play a crucial role in regulating WSC storage. We, therefore, propose that the patterns of WSCs are important aspects of plants ecological strategies particularly in regions subject to periodic N stress. Further, the mechanistic or genetic linkages of WSCs with other traits (such as V_{MASS}, RS_R, FL_W, RUE, and N_{LA}) in genetically different wheat species deserved further research.

3.5.4 Conclusions

Conferring theoretical WSCs gains from the overexpression of any trait might be questionable due to the existence of compensatory mechanisms (such as N metabolism) as well as largely unestablished mechanistic or genetic linkages among traits. Yet, trait information might be useful for developing strategic crosses based on the theoretical combination of selected traits. Overall, the data subjected to the combined analysis of correlations, univariate, multivariate regressions and path analyses suggests that high WSC trait in wheat under three N levels is primarily associated with V_{MASS}, RS_R, FL_W, RUE, and N_{LA}. In addition, flowering time greatly influenced all the traits, including WSCs. Therefore, these traits could be used as WSC markers to prescreen a large number of genotypes/germplasms to derive genotypic variation for WSC. Such an integrated knowledge would greatly enhance our chances of achieving genetic improvement in WSCs and, therefore, higher crop yields.

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Chapter three described the traits regulating total water-soluble carbohydrate storage in wheat plants. However, the regulation of traits varied depending on the N levels. Simulating total WSCs accumulation under different N levels may allow wide extrapolation of these results to other N levels, therefore, it is possible to predict total WSC storage and grain yields.

Chapter four will describe the simulation model for total WSC accumulation under different N levels. Although, the developed model predicted total WSC accumulation with acceptable RMSE, it fairly suggests that there is enough room for further model improvements.

Our understanding of a biological phenomenon is incomplete unless we can relate it to (or translate it into) phenomena in the adjoining levels of the organizational scale through modeling.

Passioura, 1979

Chapter Four

SIMULATION MODELING OF WATER-SOLUBLE CARBOHYDRATES UNDER THREE NITROGEN LEVELS

This paper will appear as

Ravi Valluru, Johanna Link, Wilhelm Claupein. Simulation modeling of water-soluble carbohydrate accumulation under different nitrogen levels. (In preparation)

Modeling Water-soluble Carbohydrate Accumulation

Keywords

Phonological model, simulations, thermal degree days, water-soluble carbohydrates,

4.1 Abstract

Physiological models are commonly used to reveal agronomic questions. Crop models, therefore, are increasingly seen as a useful tool for comprehending complex relationships between plant physiological process and environments. Many growth models provide, as of now, insights into the mechanisms of plant development by incorporating physiological processes, such as the transport and allocation of carbon. Here, I developed a simple phenological model for carbon accumulation, in the form of water-soluble carbohydrates, during vegetative period in four wheat genotypes. I integrated and evaluated this model under crop management factors such as low (0 kg ha^{-1}), medium (100 kg ha^{-1}), and high (200 kg ha^{-1}) nitrogen supply. The proposed model predicted higher rate of WSC accumulation in the early stages of crop growth and lower rates in the later stages. Overall, the model predicted the rate of WSC accumulation with a RMSE of 6. 58, suggesting that the proposed model simulated well. Nevertheless, the predicted rate of WSC accumulation was close to the observed data only in low and high N level, and more deviated in medium N level. The model predicted total WSCs well with the observed data, however, overestimated it at early stages and underestimated it at later stages, largely due to respective rate of WSC accumulation. Under different N levels, the total WSCs were 11%, and 17% higher than the observed WSCs in low and medium N levels, respectively. In high N level, however, the model predicted 12% lower total WSCs. Overall evaluation of the model with the predicted dataset indicated that the prediction errors for the rate of WSC accumulation were more, and RMSE were mostly obtained between 20-30% in all N levels. For total WSC accumulation, the prediction errors were less, and the RMSE was mostly less than 20% in all N levels.

4.2 Introduction

Crop production is a complex process. The simulation and modeling of crop systems has been proposed as a broad field (Sinclair *et al.*, 2004). Crop models have been used, for example, to optimize agronomic management strategies and to design sustainable agro-ecosystems (Kropff *et al.*, 2001). Capturing the key physiological processes through simple relationships might assist in identifying factors limiting crop yields. This explicitly allows exploring the potential uses of whole-crop physiology models in various aspects of genetic analysis (Yin *et al.*, 2004) including identification of main yield determining traits (Bindraban, 1997); defining optimum selection environments (Aggarwal *et al.*, 1997); evaluation of selection efficiency (Chapman *et al.*, 2003); design of crop ideotypes for a target environment (Kropff *et al.*, 1995); and assisting multi-environment testing (Dua *et al.*, 1990).

Process-based models[1] are the best tools to describe a complex process such as plant physiological processes. Since plant responses to external factors occur at different scales in both time and space, it would be worthwhile to employ a variety of models. These models synthesize isolated empirical and theoretical results and interpret them from various parts of physiological processes, and process responses predicted by the models represents the best estimates that current knowledge can supply. These projections will, at least in part, be open to test by experimentation and monitoring of selected plant systems.

[1]Process-based modeling approach provides means to combine various physiological processes which result in structural growth.

Physiological crop models are commonly used to answer agronomic questions (Brisson *et al.*, 2002). Several wheat simulation models, including CERES-Wheat (Ritchie *et al.*, 1985), SWHEAT (van Keulen and Seligman, 1987), AFRCWHEAT2 (Porter, 1993) and APSIM-N wheat (Asseng *et al.*, 2002) thus far, have been developed to predict the development, accumulation, partitioning and remobilization of biomass (C, N) in wheat. However, few studies have attempted to quantify the dynamics of carbon flow in plant and protein accumulation in grain of wheat (Martre *et al.*, 2003; 2006; Pan *et al.*, 2007).

Based on the results of several experiments under semi-controlled and field conditions, Triboi *et al.* (2006) unravelled the complexity of the relationship between grain yield and protein concentration under variable climatic and soil

conditions, and discussed some of the possibilities to break these genetic negative relationships. Asseng and Milroy (2006) explored the capacity of the APSIMN-wheat simulation model to mimic the expected or published qualitative relationship between grain yield and protein concentration under particular environmental and management stresses and with a changing genetic yield potential. New algorithms to simulate grain mass and grain protein concentration within the framework of the widely used wheat simulation model CERES-Wheat were proposed by Weiss and Moreno-Sotomayer (2006).

However, wheat quality depends not only on protein fractions, but also on several complexes such as starch. The distribution and size of a single starch granule, percentage of damaged starch and the ratio of amylose to amylopectine are important aspects of wheat grain quality. Besides, fibers such as arabinoxylanes and glucans, lipids, minerals and vitamins also play crucial role in grain quality. Modelers have not paid much attention on these grain components, partially due to more focus on grain yield; and largely also due to lack of sufficient information to develop and evaluate such models.

Apart from starch as a single unit, which represent 70-80% of the final grain dry mass, the dominant forms of carbon or its compounds that build up or convert into a starch granule and the quantitative and qualitative variations of these starch components remains to be determined. For example, dataset is still lacking to develop a mechanistic modeling framework of grain pentosans, minerals or vitamins in response to externally-driven factors. Besides, carbohydrate fractions such as fructans, the dominant reserve carbohydrate in wheat, are yet to be included in these models. Perhaps, the development of such data sets required strong collaborations between agronomists, crop physiologists and modelers.

However, using simple data sets, mathematical algorithms can be derived that can potentially represent a simple physiological phenomenon such as carbohydrate accumulation. Given the importance of temperature in wheat growing conditions, which significantly influence the wheat grain end-use quality, temperature sum can be used as a driving factor for the development of the models. Recently, Spiertz *et al.* (2006) analysed the stability of grain dry mass, protein concentration, glutenin macro polymer fraction, glutenin particle size, and particle size distribution, of three genotypes of spring wheat with different temperature tolerance, grown at two temperatures and exposed to short spells of

heat during the early phase of grain filling. Dupont *et al.* (2006) analysed the interactions between post-anthesis temperature and N nutrition on the accumulation and composition of storage proteins in wheat grains and baking and mixing quality, giving new insights into the regulation of the proteomic and transcriptomic expression of storage proteins. In similar lines, here, we developed a phonological model based on temperature sum for the accumulation of water-soluble carbohydrates during vegetative period in wheat. Here, we assumed that all the physiological processes responsible for the production and regulation of WSCs is fairly, indeed to large extent, regulated or dependent on the temperature of the growing conditions. Such a model usually does not represent completeness of the process of interest, but may only provide a comprehensive first step towards understanding of the process, which might be helpful for development of more robust mechanistic models.

4.3 Model description

4.3.1 General model description

The plant is divided into three parts: (1) leaf, *l*, (2) root, *r*, and (3) stem, *s*. The leaf includes both blade and sheath. The subscript *i* is used for all three components, i.e. i = *l, r*, and *s* unless otherwise specifically indicated. The state of total dry matter in each component at a given time, *t*, consists of three state variables: S_i (g CH_2O m^{-2}), C_i (g CH_2O m^{-2}) and A_i (g substances m^{-2}). S_i is C_6 sugars, C_i is cell-wall compounds such as cellulose and hemi-cellulose and A_i is the rest of the chemical substances such as minerals, proteins, starch and other metabolites. The dry weight of each component, W_i, can be written as

$$W_i = S_i + C_i + A_i \qquad \text{(eq. 1)}$$

It is assumed that C_i and A_i is a function of W_i,

$$C_i = \frac{g_i - 1}{g_i} W_i, \qquad \text{(eq. 2)}$$

$$A_i = \frac{g_i - 1}{g_i} W_i \qquad \text{(eq. 3)}$$

where g_i is a coefficient and ≥1. If g_i = 1, then C_i and A_i become 'zero', indicating that total dry weight consists of only sugars. Xue *et al.* (2008) found that cell-wall compounds (cellulose and hemi-cellulose) are inversely associated with total water-soluble carbohydrates in wheat. They found that a wheat plant with a unit WSC (mg g^{-1}) may contain 2.22 mg g^{-1} cell-wall compounds. If the total WSC concentration varies with total dry matter, the g_i for above-ground parts can be derived as

$$g_i = \frac{W_i}{a_{g_i} W_i - b_{g_i}} \quad \text{(eq. 4)}$$

where a_{gi} and b_{gi} are constants. If $W_i \leq 1$ g m^{-2}, g_i = 35.07. Substituting eqn. (2 & 3) into eqn. (1) gives,

$$W_i = g_i S_i \quad \text{(eq. 5)}$$

Thus, the dry weight of i is expressed as the sum of total dry weight of sugars with a multiplier of g_i. The concentration of WSCs (C_{si} mg g^{-1}) is defined as

$$C_{si} = \frac{S_i}{W_i} \quad \text{(eq. 6)}$$

Combining eqn. 5 & 6 gives:

$$g_i = \frac{1}{C_{si}} \quad \text{(eq. 7)}$$

The concentration of WSCs in above-ground parts varies with time of year and developmental stages. The variables S, C, and A at a given time, t, thus become derivative of W_i. This indicates that the accumulation of sugars, cell-wall compounds and other substances is simply in proportion of total dry matter accumulation in the plant. However, it is considered that senescence is not an inducing factor for either total dry matter or WSCs during vegetative period.

4.3.2 Model for water-soluble carbohydrate accumulation

The process of WSC synthesis and its consecutive accumulation in plants is considered to occurr via three consecutive phases (Fig. 1). During initial period of seedling establishment, total WSCs in a plant are relative small (about 35.07 mg g^{-1} DW), presumably due to utilization for seedling survival and plant establishment.

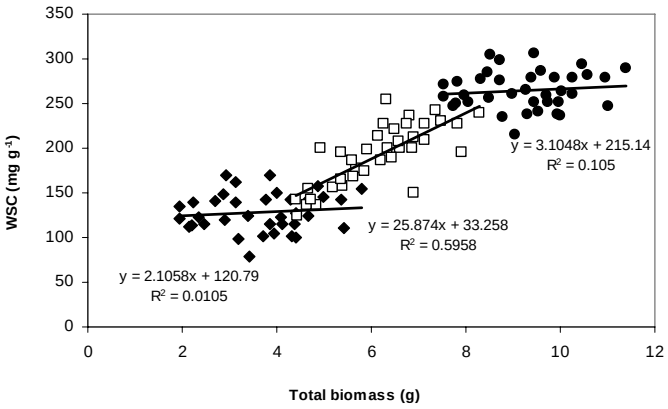


Figure 1 The pattern of total WSC accumulation in relation to total dry matter during vegetative period in wheat.

During the second, 'linear phase', the increase in total WSCs is rapid and linear, extending through most of the vegetative period, including stem elongation, during which the WSC accumulation rate is maximum and almost constant. During the third, 'plateau phase', the rate of WSC accumulation declines and remians almost constant (Fig. 1).

The total WSC accumulation in a plant can be determined as

$$\begin{aligned} WSC_i &= WSC_0 + RWSC_1 && i = 1 \\ &\quad WSC_{i-1} + RWSC_i && i > 1 \end{aligned} \qquad \text{(eq. 8)}$$

where WSC_0 is the initial WSC content (mg g^{-1} DW), WSC_i and WSC_{i-1} are total WSC accumulation values on day i and day i = 1 of the vegetative period. $RWSC_1$ and $RWSC_i$ are the rates of total WSC accumulation on day 1 and

day i (mg g^{-1} day^{-1}). The $RWSC_i$ is determined by the availability of carbon source in the plant and the capacity of WSC synthesis in leaf, as

$$RWSC_i = (RWSC_m - f(A_i)) - 0.001892 \times ACC_i / f(N_i) \qquad \text{(eq. 9)}$$

where $RWSC_m$ is the maximum rate of WSC accumulation (mg g^{-1} day^{-1}). This character fairly varies within genotypes, and is altered by external environmental factors such as temperature ($f(T_i)$), and abiotic resource factors such as plant nitrogen availability ($f(N_i)$). ACC_i is the available carbon for carbohydrates accumulation. $f(A_i)$ is the factor of capacity for WSC (fructan and sucrose) synthesis in leaf on day i, which represents total photosynthetic carbon output and is closely related to total WSCs (Fig. 2).

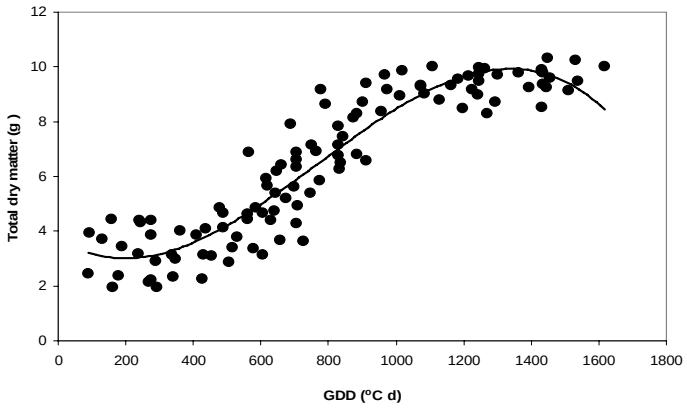


Figure 2 Response of total dry matter of a wheat plant with GDD during vegetative period.

Analyses on the experimental data suggested that the total WSC content in wheat plants was initially lower, increased gradually and descended slightly towards the end of the flowering stage during vegetative period (Fig. 3A), so the $f(A_i)$ might be non-linear with pre-anthesis degree days (GDD) (Fig. 3B). However, its factor of synthesis capacity for total WSCs follows a different trend compared to total WSC accumulation with GDD. Initially it shows an exponential growth up to the end of stem elongation period, decreased linearly after flowering (Fig. 3B).

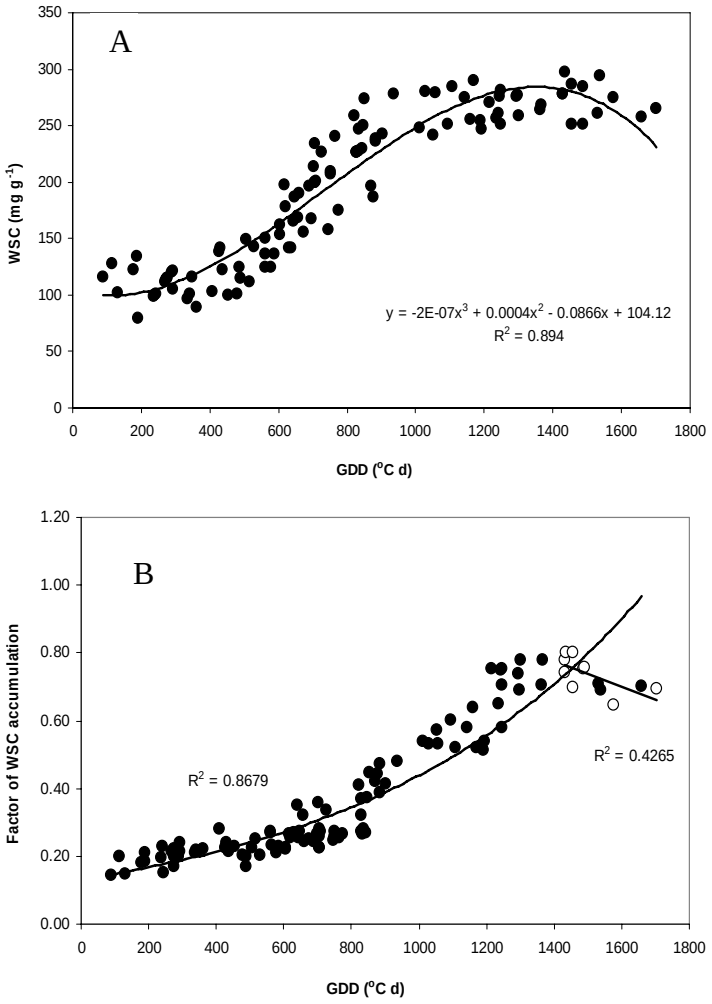


Figure 3 Response of WSC accumulation (A) and its factor of synthesis capacity (B) with GDD during vegetative period.

This can be defined as

$$f(A_i) = 0.1321 \times \exp(0.0012 \times GDD_i) \quad \text{if } GDD_i \le GDD_m$$
$$f(A_i) = 1.3067 - 0.0004 \times GDD_i \quad \text{if } GDD_i > GDD_m \qquad \text{(eq. 10)}$$

where GDD_i is the pre-anthesis growing degree days (with 0 °C base temperature) on day i; GDD_m is the accumulated growing degree days when $f(A_i)$ attains the maximum. From our experimental data analysis, it can be valued as 1400-1425 °C d.

4.3.3 Incorporating nitrogen function

In eqn. (9), $f(N_i)$ represents the impact of plant nitrogen (N) status on the rate of WSC accumulation. Plant nitrogen is inversely associated with total WSC accumulation (Wang and Tillberg, 1996; Van den Ende et al., 1999; Wang et al., 2000; Morcuende *et al.*, 2004; Shiomi *et al.*, 2006; Ruuska *et al.*, 2008). N stress strongly stimulates fructosyltransferases (FTs, fructan synthesizing enzymes, 6-SFT transcripts in barley leaves, Wang *et al.*, 2000; 1-SST activity in chicory, Van den Ende *et al.*, 1999). The enhanced FTs may regulate fructan accumulation either directly or via downstream effects on the plant growth or metabolism under N stress (Wang *et al.*, 2000).

The function of plant nitrogen can be described as:

$$f(N_i) = 1.0 - (CPN_i - APN_i / CPN_i - MPN_i) \qquad \text{(eq. 11)}$$

In the above equation, APN_i is the actual plant nitrogen concentration on dry weight basis (%) and obtained by N analysis. MPN_i is the minimum plant nitrogen concentration and set as 50% of CPN_i; CPN_i is the critical plant nitrogen concentration and determined by the following equation:

$$\begin{aligned} CPN_i &= 1.20\% \quad \text{if WSC} \geq 150 \text{ mg plant}^{-1} \\ CPN_i &= 0.60\% \quad \text{if WSC} \leq 150 \text{ mg plant}^{-1} \end{aligned} \qquad \text{(eq. 12)}$$

4.3.4 Carbon flow and availability for water-soluble carbohydrate accumulation

Plants synthesize hexose carbohydrates through photosynthesis. Some of these carbohydrates are utilized for metabolic activities and the excess carbohydrates are stored in vegetative organs. The available carbon for carbohydrates (ACC_i) in eqn. (9) is dependent on the carbohydrate utilization by the plant. The total WSCs on day i can be described as

$$ACC_i = WSC_i \times \sigma \qquad \text{(eq. 13)}$$

where σ is the ratio of relative molecular masses of carbon to hydrogen and oxygen (12/30 = 0.4).

4.3.5 Model validation

For model validation, the predicted results were compared with the field measurements to evaluate reliability and accuracy of the model output under different nitrogen conditions. The following statistical equation, root mean square error (RMSE) was used to calculate the fitness between the estimated results and observed data (Michele et al., 2003).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(P_i - O_i)^2}{n}} \quad \text{x} \frac{100}{\bar{O}} \qquad \text{(eq. 14)}$$

where *Pi* and *Oi* are predicted and observed values, respectively, and $\bar{O}$ is the observed mean value. The RMSE gives a measure (%) of relative difference between the simulated versus observed data. The prediction is considered excellent with the RMSE <10%, good if 10–20%, fair if 20–30%, poor if >30% (Jamieson *et al.*, 1998).

4.4 Materials and methods

4.4.1 Plant material

Four winter wheat (*Triticum aestivum* L.) genotypes, Tommi, Biscay, Cetus, Contra were evaluated. The genotypes have different phenotypic characteristics varying in growth parameters, yield attributes and protein contents (Ward *et al.*, 2008; Weber *et al.*, 2008). Old genotypes differ in tillering capacity and maturities (Bohn *et al.*, 1999). Primitive species differ in plant sizes; *T. monococcum* (diploid species) has long internodes with narrow leaves, whereas *T. dicoccum* (tetraploid species) has short internodes with wider leaf blades. Also,
they differ in soluble sugars (Erdei *et al.*, 2002), protein contents (Hidalgo and Brandolini, 2008), soil nitrogen uptake (Trčková *et al.*, 2005), and growth and development (Biswas *et al.*, 2008).

4.4.2 Data for model generation

Seeds of all genotypes were treated at 4 ^{0}C (42 d) to fulfil vernalization requirement (Purvis and Gregory, 1952). Seedlings of about 2-3 cm were transplanted (1 April 2008) in square-shaped plastic pots (15 x 15 x 15 cm^3, 3 seedlings per pot), each filled with 4 kg mixture of sterile soil, organic matter, and sand (4:2:1 v/v), and placed in the glasshouse. On the day of complete emergence of first leaf (after 5-7 d), all pots were moved to field conditions. The experimental design used was a completely randomized block with five replications. Each pot represents a single block that contains three plants, and 180 blocks for all species were maintained. Soil moisture was maintained close to field capacity by supplying water every day. N solution was applied (0, 100 and 200 kg N ha^{-1}) at different growth stages. Nutrient solution containing P and K (10 mM) was applied regularly once in a week (100 ml pot^{-1}). Weeding was carried out regularly. The average relative humidity, solar radiation, average temperature and rainfall were 72%, 1874 J cm^{-2}, 15 ^{o}C, and 650 mm, respectively, representing optimal conditions during the crop growing period. Climatic data were obtained from the Institute of Physics and Meteorology, University of Hohenheim.

4.4.3 Data for model evaluation

Two experiments were carried out in the field sites of IhingerHof Research Station (IHO, Exp. 1; 2007-08) and Oberer LindenHof Research Station (OLH, Exp. 2; 2007-08), at the Institute of Crop Production and Grassland Research, University of Hohenheim, Germany. The field sites (IHO: 48^o 44$'$ N, 8^o 56$'$ E; OLH: 48^o 28$'$ N, 9^o 18$'$ E) have a temperate climate with cold winters and warm summers, but differ in altitudes (450 and 700 m asl at IHO and OLH, respectively). The daily mean temperatures during the crop growing period were 7.7 ^{o}C and 7.2 ^{o}C and total precipitation was 698 mm and 854 mm at IHO and OLH, respectively. The soils at both stations are loamy.

Sowings were done on 14 and 16 October 2007 at IHO and OLH, respectively. The experiments were laid out in a randomized complete block design with three replications. Each plot consisted of 40 rows (6 x 4 m^2) and 20 rows (12 x 2 m^2) with an inter row spacing of 10-15 cm at IHO and OLH,

respectively. N was applied at three levels comprising of 0 (low), 100 (medium) and 200 (high) kg ha^{-1} as ammonium nitrate in three splits; 30 and 60 kg N ha^{-1} at the beginning of the vegetative stage (Zadoks scale 21; Zadoks *et al.*, 1974); 40 and 80 kg N ha^{-1} at the start of stem elongation (Zadoks 31); and 30 and 60 kg N ha^{-1} at the end of stem elongation (Zadoks 39) at IHO and OLH, respectively. Fungicides and herbicides were used to lower incidence of fungal diseases and weeds, respectively. When plants matured, harvesting was completed on 14 August 2008 at both stations using combine harvester.

4.4.4 Trait measurements

Total biomass was recorded at two stages, namely at anthesis stage (Zadoks scale 65), and 2 weeks after anthesis (Zadoks 69). In each experiment, 180 plants were sampled at random at each stage. Dry weight of total plant biomass was obtained after oven-drying at 65 °C for 73 h. Thermal degree days were calculated as average of minimum and maximum temperature of a day, with a base temperature of 0 °C.

4.4.5 Nitrogen, carbon and carbohydrate analyses

Dried samples of leaf, stem and root were analysed for total N (Dumas, 1962) and C using a Heraeus macro-N analyzer (Hanau, Germany). Water-soluble carbohydrates were analysed only in leaf and stem samples by a modified HPLC method of Turner *et al.*, (2006). For extraction of WSCs, ground plant material was weighed in 2 ml screw-capped plastic tubes and 1.5 ml of hot deionized water was added. Tubes were sealed, vigorously shaken in a thermomix for 10 min at 85 °C and 30 min at 25 °C, and then centrifuged at 20 °C for 10 min. The aqueous extract was filtered with 0.45 µm syringe filter into HPLC vials. Sugars were separated and quantified by HPLC on a 300 x 7.8 mm column of Phenomenex Zezex RCM-Monosaccharide Ca^{+} (BISCHOFF Chromatography GmbH, Leonberg, Germany) at 85 °C with degassed water as a mobile phase, at 0.6 ml min^{-1} for separation of fructan and sucrose. Samples were analyzed using a RI-8100 refractive index (RI) monitor, 2200 series pump, AS-728 auto-sampler and high-resolution liquid chromatography instrument interface (BISCHOFF Chromatography GmbH, Leonberg,

Germany). Aqueous solutions (range 100–5000 ppm) of commercially available carbohydrates (inulin and sucrose, Sigma, Germany) were used as standards.

4.5 Model evaluation

4.5.1 Determination of the parameter $RWSC_m$

The present model incorporated, as described previously, a genotypic parameter as the maximum rate of WSC accumulation during vegetative period ($RWSC_m$ mg day^{-1}). The rate of $RWSC_m$ in different wheat genotypes would reach maximum value at the middle of the vegetative period, where the growth is in linear function, corresponding to the stage of maximum rate of stem biomass accumulation, if the total carbon available for stem biomass is adequate. All genotypes cultivated under glasshouse conditions were used for estimating the cultivar specific parameter of $RWSC_m$.

Based on this data set, the average rate of WSC accumulation was estimated and compared with the observed data (Fig. 4). This estimation predicts that, the rate of WSC accumulation was higher in the early stages of crop growth and lower in later stages. The model validation however produced a RMSE of 6.58, suggesting that the predicted values are nearly in agreement with the observed data. However, overall, the predicted rate of WSC accumulation was 31% higher than the observed data.

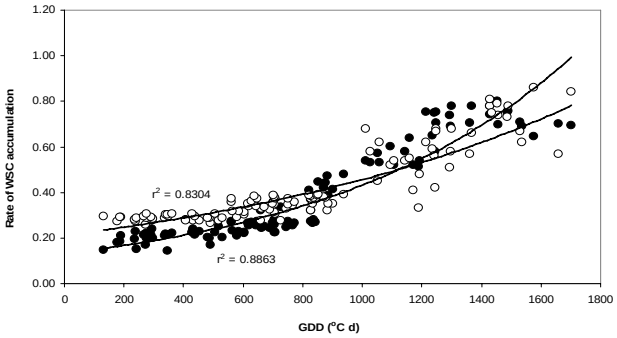


Figure 4 Rate of WSC accumulation in relation to GDD. Filled circles are observed data and un-filled circles are predicted data across genotypes and nitrogen levels.

In nitrogen non-limiting conditions, plants were still green and photosynthetically active, indicating that leaf photosynthesis provided abundant carbon source to total WSC accumulation. Nevertheless, nitrogen levels were inversely correlated with total WSC content in our study. Under nitrogen stress,

plants produce less structural compounds leading to lower biomass; and this situation initiates more carbon flux to carbon metabolism. This suggests that under nitrogen stress, limited sink capacity was not due to limited resources and the WSC accumulation rate would reach maximum values for all genotypes tested. Given these perspectives, the values of $RWSC_m$ for four wheat genotypes used in the present model were determined respectively as, 1.4 (Biscay), 1.50 (Cetus), 1.54 (Contra), and 1.59 (Tommi).

Accordingly, all genotypes showed lower rates of WSC accumulation (Fig. 5). The genotype Biscay showed lowest rate of WSC accumulation; while Tommi showed highest rate of WSC accumulation. Comparing the rates of WSC accumulation under different N levels reveal that the rate of WSC accumulation was higher in N_{100}, lower in N_0 and medium in N_{200} level. These rates are consistent with the total WSC accumulation under the respective N level. However, the proposed model predicted the rate of WSC accumulation nearly close to the observed data only in low and high N level, and deviated largely in medium N level (Fig. 7).

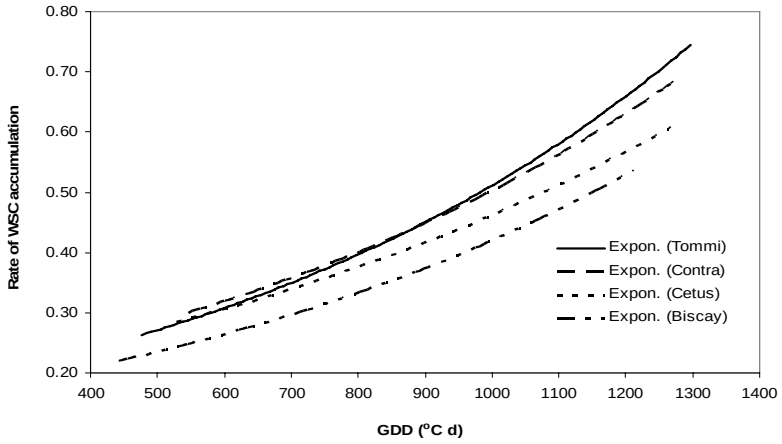


Figure 5 Rate of WSC accumulation in relation to GDD during vegetative period in four wheat genotypes. The solid, broken, dot-line, and long-dot lines indicate, respectively, the rates of WSC accumulation in Tommi, Contra, Cetus and Biscay.

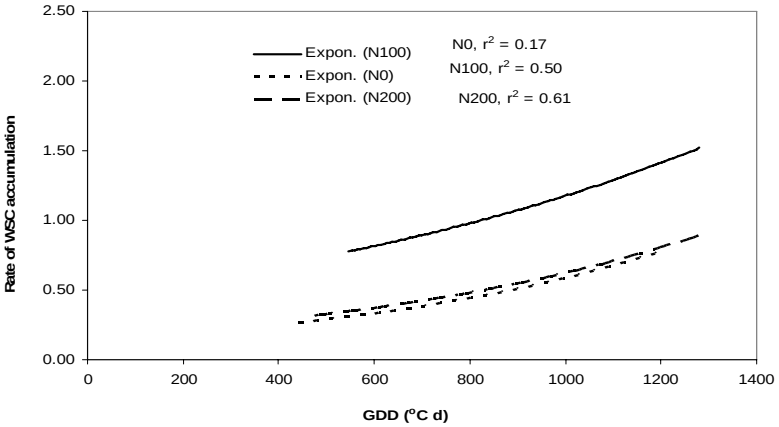
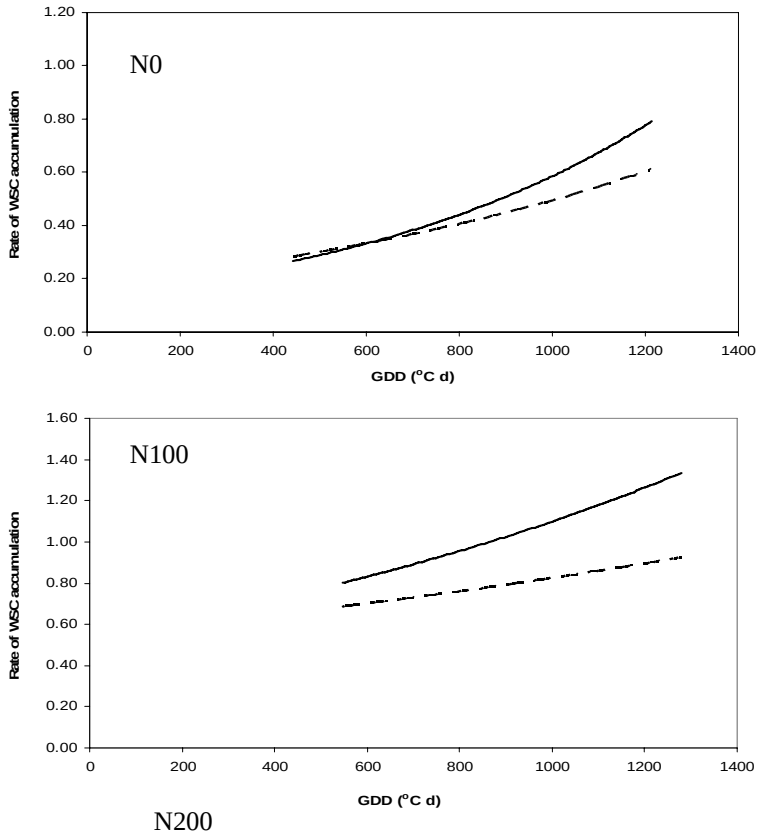


Figure 6 Rate of WSC accumulation in relation to GDD during vegetative period in three nitrogen levels across genotypes.

In low N level, the rate of WSC accumulation predicted was lower than that observed initially; however, it was high at later stages. In medium N level, the predicted rate of WSC accumulation largely deviated from the observed data set, indicating that the model is not precisely applicable at medium N level.



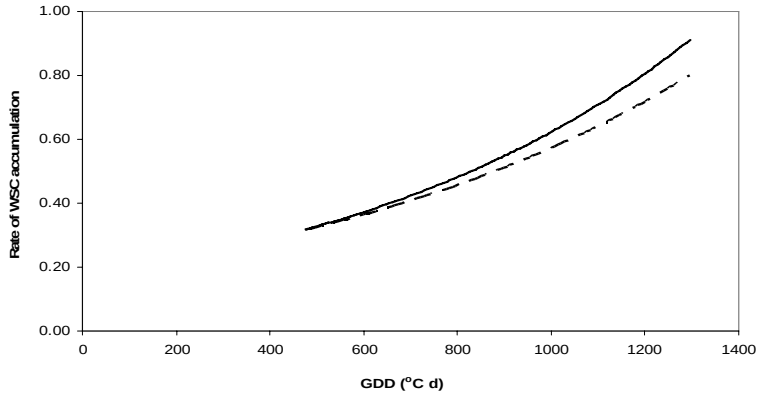


Figure 7 Rate of WSC accumulation in relation to GDD during vegetative period in three nitrogen levels across genotypes. Solid line is based on predicted data, while dashed line is based on observed data.

4.5.2 Total water-soluble carbohydrate accumulation

The model simulated the total WSC accumulation well with observations of all N levels (Fig. 8). However, the model overestimated total WSC accumulation at early stages, and underestimated it at later stages. At later stages of crop growth, the predicted values are more deviant than the observed data set. This might be due to a higher rate of WSC accumulation prediction by the model (Fig. 4). However, on average, the predicted WSCs are <1% higher than the observed values, suggesting that the model, on average, simulated total WSCs well.

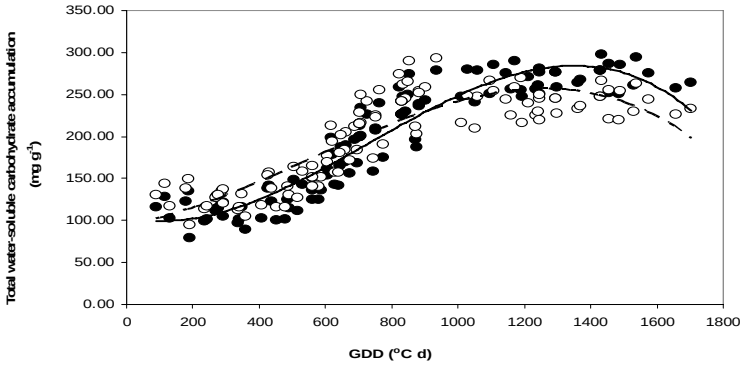
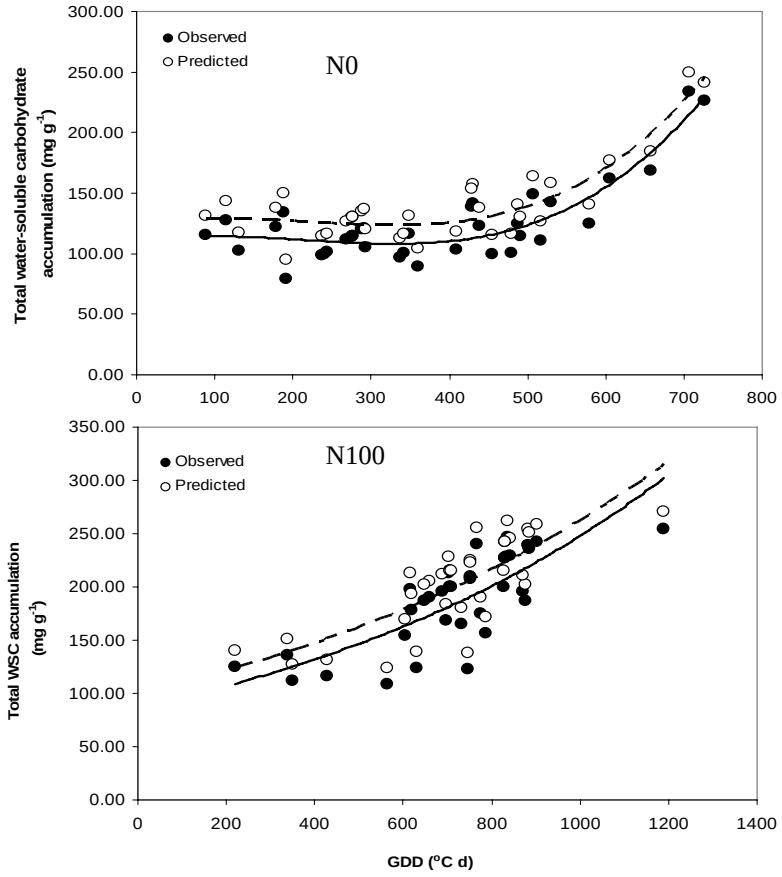


Figure 8 Total WSC accumulation in relation to GDD during vegetative period across three nitrogen levels and genotypes. Solid line is based on observed data (filled circles), while dashed line is based on predicted data (un-filled circles).

The model simulated total WSCs differently under different N levels. In all N levels, the model predicted higher WSC accumulation. However, in high N level, the model simulated lower WSCs at early stages and higher WSCs at later stages. Under conditions of different N supply, the total WSCs were 11% and 7% higher than observed WSCs in low and medium N levels, respectively, whereas in high N level, the predicted WSCs were 12% lower than the observed WSCs. This suggests that the pre-anthesis N application had marked effects on total WSCs. The duration of accumulation of these WSCs was also significantly modified by N nutrition. Overall, these variations in the kinetics of accumulation of total WSCs fractions were overestimated by the proposed model.



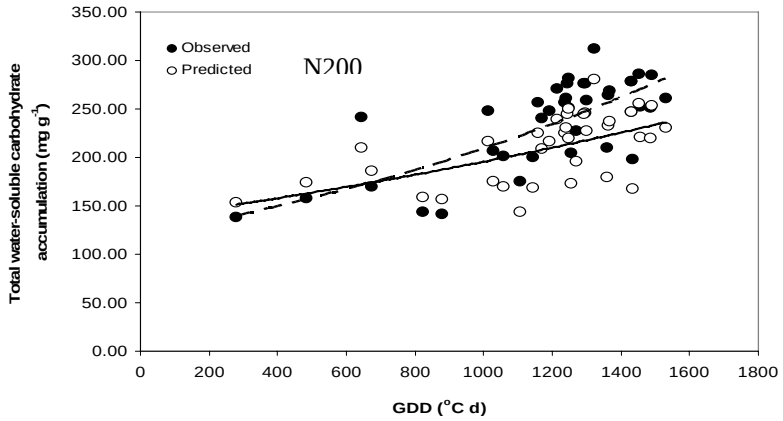


Figure 9 Total WSC accumulation in relation to GDD during vegetative period across three genotypes under three N levels. Solid line is based on observed data (filled circles), while dashed line is based on predicted data (un-filled circles).

4.5.3 Validation of the model performance

Figures 4-9 show the rate of WSC accumulation as well as total WSCs accumulation under three N levels. The rate of WSC accumulation was curvilinearly increased to the maximum (corresponding pre-anthesis GDD was 1050 °C d), and then decreased with accumulated growing degree days than the observed rate of WSC accumulation. This might be due to changing availability of carbon within the plant, and rate of WSC accumulation (synthesis of different WSC compounds such as fructans, sucrose, glucose and fructose) during vegetative period.

Evaluation of the model with the predicted data set indicated that the prediction errors for the rate of WSC accumulation were more, and the RMSEs were mostly between 20-30% in all N treatments (Table 1). For total WSCs accumulation, the prediction errors for the rate of WSC accumulation were less, and the RMSEs were mostly less than 20% in all N treatments (Table 2).

Table 1 RMSE for the prediction of the rate of WSCs accumulation in individual wheat genotypes under three N levels.

Genotypes	N rate (kg ha^{-1})			Mean (%)
	N_0	N_{100}	N_{200}	
Biscay	27.80	32.87	15.61	25.42
Cetus	34.12	32.97	13.96	27.07
Contra	51.00	31.07	19.28	33.78
Tommi	43.86	25.23	9.41	23.16
Mean	39.19	30.53	14.56	28.09

Table 2 RMSE for the prediction of the total WSCs accumulation in individual wheat genotypes under three N levels.

Genotypes	N rate (kg ha^{-1})			Mean (%)
	N_0	N_{100}	N_{200}	
Biscay	12.86	8.82	12.16	11.28
Cetus	11.96	7.40	12.88	10.74
Contra	13.88	8.40	12.13	11.47
Tommi	11.41	8.10	12.17	10.56
Mean	12.52	8.18	12.33	11.01

Among three N levels, the differences among varieties were most obvious under medium N level and smaller at high N level (Fig. 7). Under medium N level, the rate of WSC accumulation exceeded the rates of WSC accumulation under other N levels, giving more prediction errors between the simulated and observed values with the maximum RMSE, as seen in Table 1. However, the lower deviance between the simulated and observed values under low and high N levels, might have contributed to the more précised fit and reduced the prediction errors in the model. This results in the best fit between the simulated and observed values of WSC accumulation with minimum RMSE, as also seen in Table 2.

4.6 Discussion

Stem WSCs is an important agronomic trait in wheat contributing to several functions in the plant. The stored WSCs may serve majorly two important functions: providing the plants a competitive advantage; and bridging the temporal gaps that exist between resource availability and resource demand (Kleijn *et al.*, 2005). The differences in the WSC accumulation in the stems of different wheat genotypes could potentially result from the various factors such as photosynthesis capacity, carbon use efficiency, and carbon partitioning between stem reserve deposition and other physiological processes (such as maintenance respiration, growth, and cell wall synthesis). These processes primarily involve many carbohydrate metabolic genes in a number of major carbohydrate metabolic pathways. The genotypic differences in WSC accumulation could, therefore, depend on the differences in the influx and/or efflux rate of carbon into the stem sucrose pools. This suggests that the extent of WSC accumulation in wheat stems is a function of two factors: (1) the availability of carbon and the ability to store in the stem (sink strength); (2) the efficiency with which WSC are synthesized (the rate of synthesis, source capacity) (Ehdaie *et al.*, 2006).

Here, the proposed new simulation model for WSC accumulation during the vegetative period in wheat plants based on the variables such as carbon availability and degree days. The algorithms involved in the proposed model are derived through the relationships of WSC accumulation to the carbon supply strength. Figures 2 and 3 showed that the rates of WSC accumulation was non-linearly increased with accumulated GDD, and the rate of WSC accumulation increased exponentially in the early stage and then decreased linearly. The initiation of WSC accumulation was triggered by the constant rate of excess carbon availability and increased net photosynthetic rate.

Plant factors such as morphological variations and physiological processes fairly regulate total WSC accumulation in wheat plants. The increase or decrease of morphological structures, as induced by different N levels, might alter the synthesis and the accumulation of total WSCs. For example, in Chapter 3, we have identified several morpho-physiological traits regulation under different N levels, and how these morphological variations could govern WSC accumulation. Vegetative variables such as total biomass, flag-leaf width

and root: shoot ratio might correlate themselves differently, together with different correlations with

physiological traits such as radiation use efficiency and nitrogen concentration in the plant. For example, although total N concentration is negatively correlated with total WSC storage, the larger effects of radiation use efficiency might still be able to manage to provide excess carbon, thereby, accumulation of WSCs, particularly under high N levels.

Besides plant factors, environmental factors such as temperature, water status and nutritional rates are of major importance that affect the rate of WSC accumulation in wheat (Ruuska *et al.*, 2006). The present model integrated the interaction of temperature and nitrogen levels on total WSC accumulation in wheat plants, by integrating the sub-model of N. Thus the present model on WSC accumulation during the vegetative period could be suitable for application to different N levels if linked with the existing crop growth models.

The present model integrated the maximum rate of WSC accumulation that was estimated from the data of four genotypes, as a genotypic coefficient. This represents the genetic parameter to describe the observed differences in the total WSC accumulation between genotypes under different N levels. The model predicted the least maximum rate of WSC accumulation for Biscay, a genotype having lower WSC storage and medium vegetative period. The accumulation of stored carbohydrate pools in different internodes commences at different times but ceases about at the same time (Schnyder, 1993). Because, the length of the accumulation period vary greatly between the internodes, which has significant consequences on the total WSC accumulation, the duration available for each internode (stem elongation period) may determine reserve carbohydrate pools in the plant (Volence, 1986; Pavis *et al.*, 2001; Ruuska *et al.*, 2006; Ehdaie *et al.*, 2006).

The model predicted higher WSC accumulation at early stages and lower WSC accumulation at later stages (Fig. 8), which is also consistent with the rate of WSC accumulation (Fig. 4). Lower rates of WSC accumulation and total WSCs might be due to strong competition from other metabolic activities and for the constructions of structural biomass. For example, the development of floral primordia initiates during the phase of rapid vegetative growth; a special attention should be given to the stems in view of insalubrious competition for the limited resources between the vegetative (upper internodes) and floral organs in

the weeks before anthesis (Cruz-Aguado *et al.*, 1999; Foulkes *et al.*, 2007). This might fairly increase the translocation of stored WSCs for the construction of these new plant tissues and decrease the rate of total WSC accumulation in the wheat stems. Furthermore, the model did not consider the water status, if water-deficit at later stages occurs, this may also reduce total WSC accumulation as stored WSC might be utilized for developing stress resistance. How such environmental stresses, occurring either at early stages or later stages, can influence total WSC accumulation in wheat plants might be an interesting research perspective, and is described in the next chapter.

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Chapter four described a simulation model for total WSC accumulation under different N levels. The model explained total WSC accumulation with RMSE of 10-12% and total variation up to 30%. This variation can be explained by different trait regulations. However, besides environmental factors such as temperature, chilling stress can also impact the carbohydrate storage.

Chapter five will describe the effects of early chilling effects on wheat and water-soluble carbohydrate storage during recovery period. Understanding such environmental impacts on carbohydrate storage may allow better assessment of plant adaptation to abiotic stresses that allow further crop improvement.

No man qualifies as a statesman who is entirely ignorant of the problems of wheat and its cultivating environment.

Socrates

Chapter Five

WHEAT AND WATER-SOLUBLE CARBOHYDRATE PLASTICITY TO EARLY CHILLING

This paper will appear as

Ravi Valluru, Johanna Link, Wilhelm Claupein. 2009.
Consequences of early chilling stress in two primitive *Triticum* species.
(Revision submitted to Plant Biology, April 2009)

Wheat and Water-soluble Carbohydrate Plasticity to Early Chilling

Keywords

Chilling stress, morphological variation, plasticity response, reserve carbohydrates, *Triticum* spp.

5.1 Abstract

Plasticity of the phenotypic expression of two primitive wheat species (*Triticum monococcum* L. and *T. dicoccum* S.) was studied in response to early chilling stress. Selection differentials, gradients, and plasticity costs on plant growth, resource allocations, and reserve carbohydrate consumption were estimated. Regression analysis was applied to investigate differential developmental changes and paths between chilling treatments. Five-day-old seedlings of *T. monococcum* and *T. dicoccum* differing in plant stature and reserve carbohydrates were treated with early chilling temperature (4 °C) and compared with control plants grown at 23 °C. Early chilling stress resulted in a significant increase in leaf mass ratio (LMR) and relative growth rate (RGR), a reduction in flag leaf size, total biomass, specific leaf area (SLA), and reserve carbohydrates at flowering, together with an advanced onset of flowering. Selection analysis within the early chilling environment favoured early flowering, smaller SLA, higher LMR, and lower reserve carbohydrates suggesting that the observed responses were adaptive. While lower SLA may reduce the early chilling stress effects at an individual leaf level, a higher LMR and utilization of reserve carbohydrates indicated that the compensatory growth of chilled plants during the recovery period relied on the concerted action of altered resource allocations and reserve carbohydrate consumption. With selection gradient analysis, we found similar direct selection on these traits, except for SLA and sucrose indicating that these two traits have indirect effects on fitness. Thus, the total effects of SLA and reserve sucrose on relative fitness seem to be buffered via rapid growth rate in chilled plants. However, a significant cost of plasticity was evident only for flowering time, LMR and fructan levels under early chilling environment. Further, a regression of daily cumulative plant biomass derived from a crop growth simulation model (CERES-Wheat) on crop growing period revealed a divergent developmental pathway for early chilled plants. Our results showed that not only are the characteristic architectures in two *Triticum* species plastic, but the regulating mechanism of intrinsic developmental (ontogenetic) pathway is also sensitive to early chilling stress.

5.2 Introduction

Phenotypic plasticity[1] can be described as a physiological or developmental contingency of phenotypes on the environment (Bradshaw 1965, 2006). It plays an important role in the ecological breadth of plant species and acts as a source of phenotypic variation (Valladares *et al.* 2007). Plasticity studies have often focused on patterns of expression and evolution; however, understanding how phenotypic diversity is generated by the coherent change of associated traits at various levels is also essential. There is substantial development of theory and statistical approaches to allow for more integrative approaches to the study of phenotypic plasticity. Within the frame-work of the phenotypic plasticity, the environmental contingency of phenological traits often displays a set of optimal fitness responses that have largely been studied in plants (Rohde & Junttila 2008). In addition, numerous complex patterns of trade-offs and constraints are expected to occur (van Kleunen & Fisher 2005; Dechaine *et al.* 2007; Valladares *et al.* 2007), and the genetic settings underlying these are postulated to be costly (Weinig *et al.* 2006; Dechaine *et al.* 2007).

[1]Plasticity refers to the degree to which an organism's phenotype is determined by its genotype. This change is irreversible.

Plastic responses of phenotypes can be found under two cases: differences in the growth rates of plants along a fixed developmental pathway and/or differences in the patterns of trait change with phenotype size development (a different developmental pathway). However, the latter might be more ecologically important, because it implies a more complex interplay between the environment and plant development than simply a delay in growth rate alone (Moriuchi & Winn 2005; Cavallero *et al.* 2008). Empirical evidences suggest that the size of the plant in relation to the spatial scale of heterogeneity determines the likelihood and the average degree of intra-plant variation in biotic and abiotic conditions. Since, developmental processes and their interactions produce morphological variations; they encompass a wide range of processes such as developmental switches that lead to bifurcations of developmental pathways[2] or signaling between tissues through various molecular mechanisms (Wilkins 2002; Barthelemy & Caraglio 2007). Hence, developmental interactions can mediate the expression of genetic and environmental variation by transmitting their effects across different morphological traits and therefore contributes to the patterning of all

[2]A developmental pathway denotes the ensemble of processes that generate a trait. It incorporates a multitude of interacting molecular and cellular mechanisms that underpin the processes of organismal development, which can themselves be complex networks of interaction.

components of phenotypic (co)variation among traits (Barthelemy & Caraglio 2007).

All higher plants and their functional modules (plant parts) follow pre-programmed developmental pathways (de Kroon *et al.* 2005). Thus, a divergent developmental pathway can affect not only the shape of plants, but also alter their responses to the environment (de Kroon *et al.* 2005). This phenomenon, termed 'developmental (ontogenetic) contingency' (Diggle 1994; Watson *et al.* 1995), has been emphasized a long time ago but has only recently been explicitly implemented in the concepts of phenotypic plasticity (Wright & McConnaughay 2002; Niinemets 2004; Moriuchi & Winn 2005). 'Developmental reaction norms' (DRN), a concept developed a decade ago (Schlichting & Pigliucci 1998) is an attempt to include 'ontogeny'[1] as one of the main axes determining the outcome of plasticity studies. Later, studies have found that developmental pathways can only be altered by environmental cues early in life (Gedroc *et al.* 1996; Weinig & Delph 2001), suggesting a possible constraint on the adaptive phenotypic plasticity. The effects of interacting abiotic conditions and heterogeneity on plasticity, especially along different developmental patterns have received relatively little attention in the plasticity literature, despite the general acknowledgement on the importance of different developmental pathways as the basis for plastic responses (Wright & McConnaughay 2002; Niinemets 2004).

[1]Ontogeny describes the origin and the development of an organism from the fertilized egg to its mature form.

[2]Fitness is the capability of an individual of certain genotype to reproduce.

[3]Genetic costs are the plants inherent energy responses (costs) that maintain a specific set of genes to express a particular character under a particular environment.

[4]Maintenance costs are the energy costs for maintaining a particular transcription and expression of genes and its machinery.

Many plants normally face cold stress in temperate regions, and the plants ability (or lack thereof) to acquire cold tolerance is an important factor in their ecological and evolutionary dynamics. The consequences of fitness[2], benefits, and costs of cold stress are likely to be determined, in part, by whether plant (trait) responses are inducible (expressed only in response to specific stress) or constitutively expressed (Heil & Baldwin 2002; Jackson *et al.* 2004). Inducible responses are employed only when environmental cues demand and may be less costly than constitutive responses (Bergelson & Purrington 1996; Baldwin 1998). When naturally inducible responses are constitutively expressed, they may incur fitness costs because of producing additional compounds, autotoxicity or ecological tradeoffs (Purrington & Bergelson 1997, 1999). For example, adaptation to latitudinal cold gradients favoured smaller leaves, reduced leaf blade, and petiole lengths in *Arabidopsis* (Hopkins *et al.* 2008). Consistently, early bolting was favoured and imposed plasticity costs in response to cold conditions (Callahan *et al.* 2005). Further, Karpilova *et al.* (1980) found an increased number

of thylakoids, lipid droplets, and a well-developed lamellar granal structures in chloroplasts of chilled plants. These studies suggest that the plastic costs to cold gradients could originate either from genetic[3] or maintenance[4] costs or both, and were adaptive in nature (Karpilova *et al.* 1980). Interestingly, both types of costs are likely to differ between plastic and non-plastic genotypes (Callahan *et al.* 2005). Since early chilling stress show season-long negative effects on growth and development (DeRidder & Crafts-Brandner 2008), understanding how it generates phenotypic variation via influencing developmental path of a phenotype is essential not only to estimate the variation in plant adaptation across cold environmental gradients but also to predict the effects of impending climate change on plant production.

We evaluated two *Triticum* spp. under early chilling stress and determined several morphological and physiological trait responses to the past chilling stress. We also determined how traits change as plants grew and developed and whether trait interactions could explain the observed responses between two early environments. Specifically, we tested the following questions: (1) Are chilled plants likely to increase fitness by allocating more resources to leaf and root? Greater resource use efficiency, allocation to roots and storage organs, production of smaller, thicker leaves and longer tissue turnover (Chapin *et al.* 1993) are the primary characteristics expected to be favoured in stressful environments. (2) Are the generative costs of new tissues of leaves and stems increased under chilling? The resources used to construct these structures may be acquired *de novo*, derived from shifts in allocation, and increased utilization of stored resources; or altering the 'costs' of production may increase the amount of resources needed. (3) Does reserve carbohydrates support growth during the stress recovery period? When plants stay under stress conditions, stored reserves act as respiratory substrates (Xue *et al.* 2008), supply carbon skeletons and energy (Morvan-Bertrand *et al.* 2001), involved in stress tolerant mechanisms (Valluru *et al.* 2008; Valluru & Van den Ende 2008; Van den Ende & Valluru 2009), and act as osmolytes to enhance water retention (Kawakami *et al.* 2008), which may fairly impose larger maintenance costs during the stress-recovery period.

5.3 Materials and methods

5.3.1 Plant material

Experiments were carried out using two primitive wheat species, *T. monococcum* L. and *T. dicoccum* (Schrank) Schübler (Biswas *et al.* 2008). These two species are winter types, and have different morphology, *T. monococcum* (an einkorn diploid species with AA genome, 2n=14) has strong glumes, narrow leaves and larger size, while *T. dicoccum* (an emmer tetraploid species derived from hybridization of two diploid wild grasses with AABB genome, 2n=28) has short internodes, wider leaf blades and smaller size. Also, they differ in yield, growth habit and winter hardiness (Dorofeev, 1968), soluble carbohydrates (Erdei *et al.* 2002) and protein contents (Hidalgo & Brandolini, 2008). *T. dicoccum* has higher reserve carbohydrates and can support growth under prolonged stresses; while *T. monococcum* has lower reserve carbohydrates and support growth for the shorter stress periods (unpublished results). Considering different carbohydrate synthesizing capacities and winter hardiness, we selected these species to test our hypotheses.

5.3.2 Experimental design

Seeds of the two *Triticum* spp. were obtained from Plant Breeding Gene Bank, University of Hohenheim, Germany. 300 seeds from each species were placed in petri-plates (15 seeds per plate, 20 plates per species) on tissue paper and were wetted with deionized water, and maintained under room temperatures (20 °C) for seed germination. On the fourth day, all petri-plates were wrapped with aluminum foil and kept in cold chambers (KBK / LS 4600, EHRET GmbH & Co. KG, Germany), which were preset to chilling temperature (CT, 4 °C) with a photoperiod of 16/8 h light and dark, respectively. Chilling treatment was imposed as described by Purvis and Gregory (1952). Similarly, 300 seeds from each species for control treatment (NCT) were maintained under optimum growth temperatures (23 °C). The cold chamber was lit with six banks of fluorescence lights (L 30 W/25 GROLUX tubes; Fischer-JW Zander GmbH, Germany) providing photosynthetic photon flux density of 1200 µmol photons m^{-2} s^{-1} during the chilling treatment period. Cold treatment was provided for 42 d. Seed moisture content was maintained close to 50% of seed dry weight by

supplying water twice weekly. All petri-plates were randomly repositioned every week within the cold chamber to ensure equal distribution of cold units. Seeds from each species (as group) were regularly weighed twice weekly for measuring net gain in seedling weight during germination in both environments. At the end of the treatments, seedlings of approx. 2-3 cm length from both CT and NCT environments were transferred to square-shaped plastic pots (3 plants pot^{-1}, 15 x 15 x 15 cm^3), each filled with 4 kg mixture of sterilized peat, organic matter and sand (4:2:1 v/v), and placed in the glasshouse. In both experiments, both treatments (seedling environment) and species were arranged in randomized block design (RBD). Each pot represents a single block that contains 3 plants, and 200 blocks for each species with two treatments (100 blocks for each treatment) were maintained. Experiments were carried out in early summer of 2007 (Exp. 1 in April; and Exp. 2 in May) at the Institute for Crop Production and Grassland Research, University of Hohenheim, Stuttgart (48° 43' N, 09° 13' E, 407 msl) Germany.

All seedlings were grown in a naturally lit glasshouse with day and night compartments. Pots were placed on an automated mobile trolley (4 x 1 m^2) that could be drawn out of the glasshouse when necessary. Soil moisture content was maintained close to the field capacity by supplying water everyday. Nutrient solution containing N, P and K (10 mM) was applied regularly once a week (100 ml pot^{-1}). On the day of complete emergence of first leaf (approx. 5-7 d), all pots were moved and maintained under the field conditions until samplings were taken. During this period, weed control and fertilization (once in a week) and irrigation (every day) were carried out regularly. The 23 ^{0}C control treatment corresponds to the optimum growth temperature for wheat, which is slightly above the average day temperature in summer (~20 ^{0}C) in south Germany, (estimated from official long term records (>40 years) at University of Hohenheim, Stuttgart, Germany). The average relative humidity and solar radiation was 66% and 2052 J cm^{-2}, respectively, representing optimal conditions during the crop growing period.

5.3.3 Flowering pattern and growth analyses

The effect of seedling temperature treatment on flowering pattern was examined in both experiments. From the day of flag leaf emergence, plants

were closely observed for flower initiation every day. Once ear emerged (heads emerging through the slit of flag leaf sheath), days to complete flowering were counted. For phenotypic traits that exhibit marked variation throughout growth and development, comparisons at one or more common points in ontogeny may be most relevant. Thus, three samplings at three different stages were taken. Beginning 48 d after treatments (approx. growth stage 12 on the Zadok *et al.* 1974 scale), 50 randomly selected plants across blocks per each treatment were harvested. The second harvest was done four weeks before flowering (4WBF; stage 40), and consequently the third sampling at flowering (FT, stage 69). In the first sampling, only biomass was measured. To estimate carbon turnover rates, the dates of appearance of the first true leaf and its complete browning (the first date on which a leaf was completely brown) were recorded for each seedling. Before each destructive sampling, the area of the youngest, fully expanded leaf for each harvested plant was measured to calculate leaf length and size. Each plant was separated into leaf, stem, root and ears. Plants were weighed to obtain dry biomass after drying at 65 °C for 72 h.

Chilling stress effects on plant growth and development have often been attributed to reductions in net assimilation capacity (Allen & Ort, 2001) via stomatal and non-stomatal effects on photosynthesis (DeRidder & Crafts-Brandner, 2008). The altered assimilation capacity may change growth responses of the plants via altered growth rates and resource allocations. Thus, in our experiments, specific leaf area (SLA) as the ratio of fully expanded leaf lamina area to its dry mass, leaf mass ratio (LMR) as the ratio of leaf dry weight to total plant dry weight and net assimilation rate (NAR) as the increase in plant mass per unit leaf area and time were calculated. Based on these parameters, relative growth rate (RGR) was calculated (as a product of NAR* SLA* LMR) (Atkin *et al.* 2006). Further, we quantified percent allocation to leaves, stems and roots for each individual as the ratio of the dry weight of each structure to total dry weight.

5.3.4 Carbohydrate analysis

In addition to altered growth and allocations, the perturbations in carbohydrate and amino acid metabolism and oxidative damage have been implicated as limiting factors to growth during post-chilling stress recovery (Van Heerden et al. 2004). Plants utilize more reserve carbohydrates under stress conditions for

metabolic maintenance and stress resistance, and thus affect storage capacity. Accordingly, we measured water-soluble carbohydrates (WSCs) by high-performance liquid chromatography (HPLC). For extraction of WSCs, grinded plant material was weighed in 2 ml screw-capped plastic tubes and 1.5 ml of hot deionized water was added. Tubes were sealed, vigorously shaken in a thermomix for 10 min at 85 °C and 30 min at 25 °C, and then centrifuged at 20 °C for 10 min. The aqueous extract was filtered with a 0.45 µm syringe filter into HPLC vials. Sugars were separated and quantified by HPLC on a 300 x 7.8 mm column of Phenomenex Zezex RCM-Monosaccharide Ca^{+} (BISCHOFF Chromatography GmbH, Leonberg, Germany) at 85 °C with degassed water as a mobile phase, at 0.6 ml min^{-1} for separation of fructans and sucrose. Samples were analyzed using a RI-8100 refractive index (RI) monitor, 2200 series pump, AS-728 auto-sampler and high-resolution liquid chromatography instrument interface (BISCHOFF Chromatography GmbH, Leonberg, Germany). Aqueous solutions (range 100–5000 ppm) of commercially available carbohydrates (inulin and sucrose, Sigma, Germany) were used as standards. Data on carbohydrates were analyzed to determine the stored carbon reserves under two early seedling environments and estimated the consumption of carbon reserves for each species as:

$$\text{Carbohydrate reserve use} = [X]_{\text{flowering}} - [X]_{\text{pre-flowering}}$$

Where [X] is the concentration of total water-soluble carbohydrates (fructan and sucrose) either before ($[X]_{\text{pre-flowering}}$) or after ($[X]_{\text{flowering}}$) flowering. All concentration values were based on a single composite sample taken from all seedling fractions at both developmental stages.

5.3.5 Data analysis

A multivariate analysis of covariance (using the MANOVA procedure of Genstat version 10.1, Lawes Agricultural Trust, UK) was performed to test for the effects of chilling treatment, species and plant biomass on flowering time, leaf number and flag leaf size. The data for these analyses were the species means estimated across blocks, so there was no block effect in the MANOVA. We elected to use MANOVA approach to account for the possibility that the

response variables of interest were correlated (Scheiner 2001). For significant overall main effects, individual univariate ANCOVAs were performed to investigate how variation in each trait is affected by each independent variable. In the analysis, both species and treatments were considered as fixed effects. Whole plant biomass was used as a covariate. When the interactions between treatment and covariate were significant for a response variable, a regression with natural log transformed plant biomass (an estimate of fitness) as the regressor was done. These regressions were done within each treatment to test if the phenotypic changes in a trait and thus the developmental pathway results in significantly increased success. However, we propose that the fitness itself is a plastic trait on the developmental pathway of a phenotype. Given this perspective, a different developmental pathway was derived by regressing daily cumulative plant biomass (fitness) on crop growing period. Since we have measured total biomass only at three developmental points of growth, we used a crop growth simulation model (CERES[1]-Wheat; Bannayan et al., 2003) to predict the total biomass on a daily basis. This allowed us to calculate total biomass of two species on a daily basis up to the flowering time, which we used for regression on crop growing period.

[1]CERES is a crop simulation model. This is an integrated part of DSSAT (Decision Support System for Agrotechnology Transfer) models, which deals the management strategies for several crops, including wheat.

To evaluate total and direct selection on the traits, selection differential and gradients were calculated for each treatment separately. To estimate relative fitness within each treatment, the species mean for log-transformed total biomass (an estimate of fitness) was divided by the within-treatment grand mean (Rausher 1992). This estimate of relative fitness was regressed on a focal trait to estimate a standardized selection differential. Selection gradients were estimated separately using multivariate regression analysis. Direct selection gradients were calculated as a partial linear regression coefficient of relative fitness on the standardized traits. Tests were conducted with and without a sequential Bonferroni adjustment[2] for multiple tests (Day & Quinn 1989). To test for the costs of plasticity, we performed a multiple regression as suggested by Dewitt *et al.* (1998) using the following model:

[2]Bonferroni adjustment is a multiple comparison correction used when several dependent or independent statistical tests are being performed simultaneously, which lower alpha value.

$$W = X + plX \quad \text{(eqn. 1)}$$

where W is the relative fitness within a treatment environment, X is the trait mean within that environment, and plX is the mean plasticity for that trait. Trait plasticities were calculated as absolute difference in trait means between chilling and non-chilling treatments. A negative regression coefficient for the plX term indicates a cost of plasticity, namely a lower fitness of plastic species relative to

nonplastic species. A positive coefficient indicates a cost of homeostasis (Dorn *et al.* 2000). We analyze the data both with and without a sequential Bonferroni adjustment for multiple tests on multiple traits (Relyea, 2002).

5.4 Results

5.4.1 Phenology, growth and developmental responses

Chilling temperature led to earlier flowering in both *Triticum* species. Flowering occurred as early as 109 d and as late as 124 d following the chilling treatment, and the greatest delay in flowering occurred in the non-chilling treatment. The differences in days to flowering between treatments were higher for *T. monococcum* that flowered 15 d earlier than *T. dicoccum* (Fig. 1; Table 1). The two species exerted significant effects on flowering time. Figure 1 depicts the mean reaction norm for phenology traits for the two species. Chilled plants also showed lower leaf number. Similarly, flag-leaf size was reduced substantially under early chilling treatment (Fig. 1; Table 1).

The relative growth rate of plants under early chilling temperatures was accelerated compared to plants grown under non-chilling temperatures (Fig. 2; Table 2). Treatment effects were more evident when comparisons were made for species of a common plant biomass. For example, the RGR of plant biomass of both species was *c.* 19% higher in chilling treatment than non-chilling plants of similar biomass. If the growth rates between pre-flowering and flowering period was compared, both species had *c.* 21% higher RGR with chilling treatment compared to their counterparts. However, we did not find significant changes in growth rates between the two species (Table 2).

Table 1 Multivariate analysis of variance results, including *F* statistics for individual ANCOVAs for three phenological traits.

Factor	MANOVA		Univariate ANOVAs					
	Wilks lambda		Flowering time		Leaf number		Leaf size	
	df	F-value	df	F-value	df	F-value	df	F-value
Treatment	3	37.14***	1	2295.22***	1	498.40***	1	1215.38***
Species	3	32.43***	1	59.25*	1	0.91	1	20.97***
Plant biomass	9	31.57***	1	57.73**	1	3.38*	1	65.43***

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

5.4.2 Resource allocation responses

Consistent with the size of both species, early chilling temperature reduced whole-plant biomass significantly ($P < 0.001$) compared to non-chilling conditions (Fig. 3). On average, the decrease in total plant biomass for early chilled plants was 18 and 24% at pre-flowering and flowering compared to control plants. On average, both species grown under early chilling conditions allocated 18 and 13% more biomass to

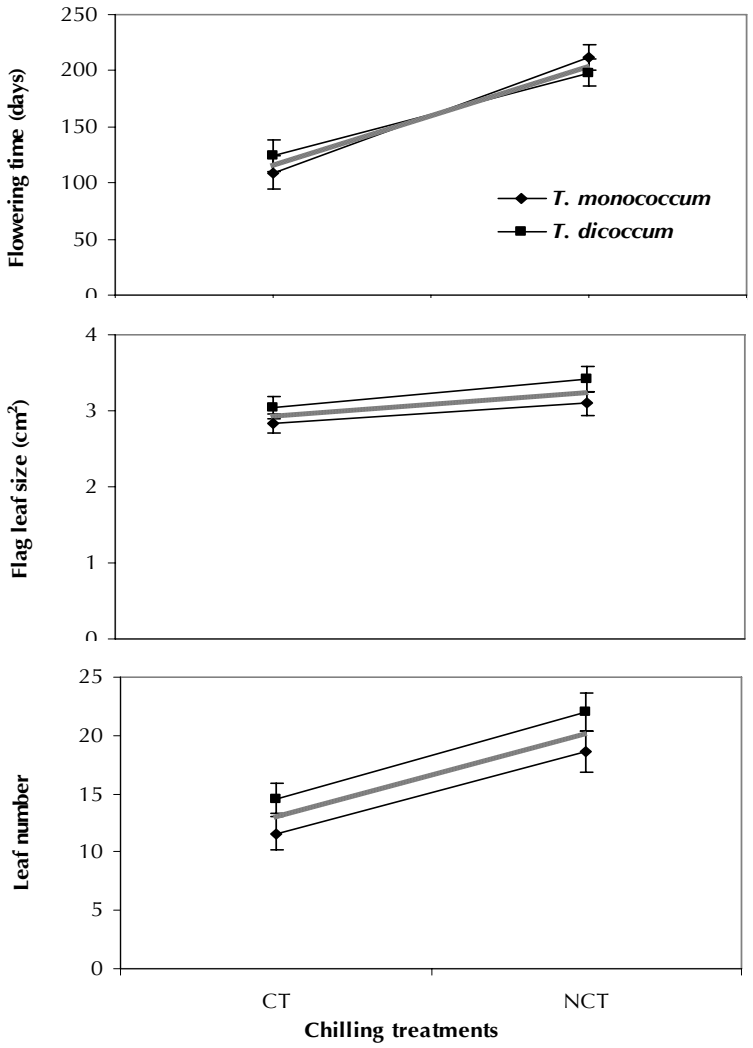

Figure 1 Flowering time, flag leaf size and leaf number variations between two early seedling temperature treatments for two *Triticum* species. Mean reaction norms for the same phenology traits are indicated by the grey bar. CT, chilling temperature; NCT, non-chilling temperature.

Table 2 Plasticity in three growth indicator traits and two reserve carbons across two *Triticum* species in response to early seedling temperature treatments. Traits were analyzed using ANCOVA with treatments (chilling and non-chilling) as main effects and total plant biomass (as a covariate).

Trait	Source of variation	df	F-value
SLA	Treatment (T)	1	7.21***
	Plant biomass (M)	1	4.88***
	T x M	1	2.06*
LMR	Treatment (T)	1	8.40***
	Plant biomass (M)	1	1.24**
	T x M	1	0.27**
RGR	Treatment (T)	1	12.86**
	Plant biomass (M)	1	1.20*
	T x M	1	0.32*
Sucrose	Treatment (T)	1	6968.69***
	Plant biomass (M)	1	12.00**
	T x M	1	2.29***
Fructan	Treatment (T)	1	1105.05***
	Plant biomass (M)	1	152.18***
	T x M	1	18.85***

Table 3 Results of selection analyses regressing an estimate of relative fitness in several phenology, allocations, growth indicators and reserve carbons in across two Triticum species in response to two early seedling temperature treatments. Costs were estimated by regressing species relative fitness within a treatment on the on a focal trait to estimate standardized selection differential. Direct selection gradients were calculated as a partial linear regression coefficient of relative fitness on the standardized traits.

Trait	CT		NCT	
	Selection differential	Selection gradient	Selection differential	Selection gradient
Flowering time	-0.27^{b}	-0.64^{ab}	-0.41^{b}	-0.66^{ab}
Flag leaf size	0.02	-0.01^{b}	0.15	0.10
Leaf number	-0.06	-0.51^{ab}	-0.11	-0.63^{ab}
Allocation to				
leaf	-0.49^{b}	-0.56^{ab}	0.02^{b}	0.21^{ab}
stem	0.01	0.32	0.03	0.04
root	-0.04	0.22	0.02	0.15
SLA	-0.37^{b}	-0.41	-0.41^{c}	-0.82^{b}
LMR	-0.36^{ab}	-0.71^{ab}	0.30	0.47
RGR	-0.64	0.91	0.41	0.68
Sucrose	-0.59^{a}	0.72^{b}	0.65	0.79
Fructan	-0.48^{ab}	-0.64^{ab}	0.84^{ab}	0.92^{ab}

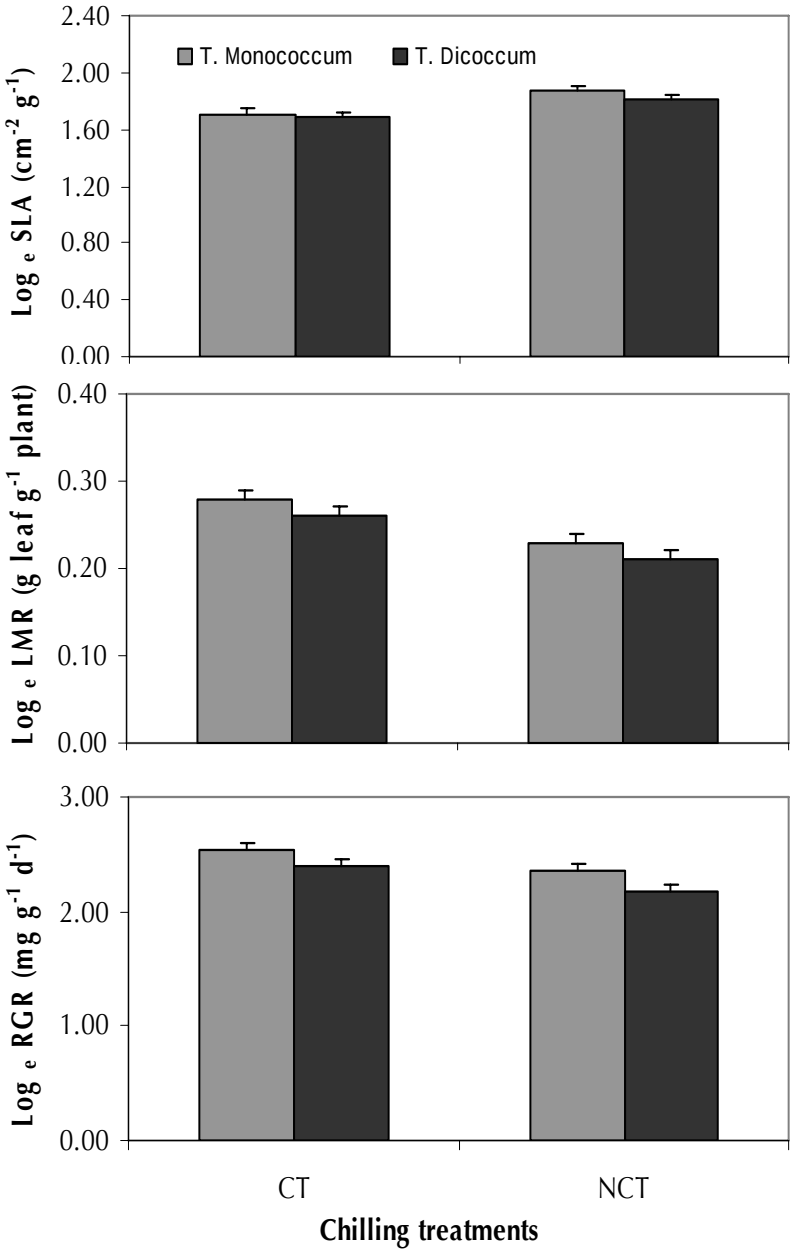

Figure 2 The relationship between log transformed means (± SE) of two *Triticum* species growth indicators, specific leaf area (A), leaf mass ratio (B), relative growth rate (C) and early seedling temperature treatments. CT, chilling temperature; NCT, non-chilling temperature.

Table 4 Costs of plasticity, as estimated by standardized regression coefficients of a species mean relative fitness on its plasticity from a within treatment multiple regression analysis.

	Selection gradient on plasticity term	
Trait	CT	NCT
Flowering time	-0.49[b]	0.22
Leaf number	-0.18	0.10
Allocation to leaf	-0.25	0.12
SLA	-0.36	0.26
LMR	-0.31[b]	0.19
Fructan	-0.21[b]	0.18

leaf and root tissue, respectively. This enhanced allocation resulted in higher LMR (*c.* 27%, Table 2). In both species, SLA decreased (*c.* 24%) with chilling treatment as plants grew larger. This indicates that, early chilling affected the allocation (LMR) and leaf properties (SLA) of both species (Fig. 4; Table 2).

In the chilling treatment, allocation to stems was reduced (*c.* 14%). Figure 3 depicts a mean reaction norm adjusted to 1 for non-chilling temperature for all allocation traits indicating the magnitude of allocational responses showing either positive (for leaf and root allocations) or negative (stem and total biomass) reactions in early chilling temperatures.

5.4.3 Reserve carbohydrate consumption

Plants grown under early chilling conditions significantly utilized both reserve carbohydrates (sucrose and fructans) in both species (Fig. 4 & 5; Table 2). On average, chilled plants were *c.* 35% deficit in reserve carbohydrates. Plants under non-chilling conditions did not use carbon reserves much, leading to lower negative values that represent higher reserve availability in the plants (Fig. 5). Suppression of leaf photosynthesis may promote more consumption of carbon reserves in chilled plants. In addition, both species had a significant difference in reserve consumption. While reserve deficit was more in *T. monococcum* (17 mg g^{-1}), *T. dicoccum* had only a reserve deficit of 13 mg g^{-1}.

Chilled plants showed more negative values for fructan contents compared to sucrose contents in both species (Fig. 5), indicating that fructans were more utilized. On average, chilling plants had *c.* 32 mg g^{-1} deficit in fructans compared to sucrose (*c.* 29 mg g^{-1}). Fructans are preferred sugar compounds that may depolymerize under stress conditions and resulting sugars may be used for metabolic maintenance during the recovery period.

5.4.4 Fitness consequences of responsive traits

Table 3 summarizes the results of selection analyses. In the chilling treatment, earlier flowering was favoured; while in the non-chilling treatment, delayed flowering was favoured. This is shown by negative selection differentials that were significant before and after Bonferroni correction. Selection gradients estimated for flowering trait also indicated that flowering in fewer days with less leaf number and size was favoured in response to early chilling.

Significantly more allocation to leaf was favoured in the early chilling, as shown by negative selection differentials and gradients. Early chilling favoured higher LMR and lower SLA. Among functional compounds, lower quantity of

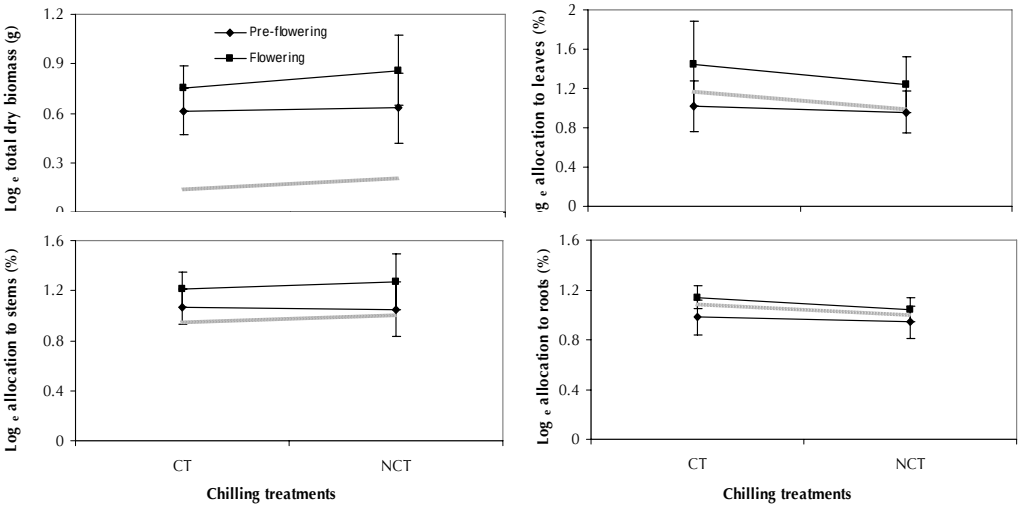

Figure 3 The relationship between log transformed means (± SE) of two *Triticum* species allocations to different structures and early seedling temperature treatments. Mean reaction norm that adjusted to 1 for non-chilling treatment is indicated by grey bar. (A) total plant dry mass shows a significant negative reaction, (B) allocation to leaves show a significant positive reaction, (C) allocation to stems show a negative reaction, (D) allocation to roots show a positive reaction in response to early chilling treatment. CT, chilling temperature; NCT, non-chilling temperature. Data were grand means of two species.

fructan was favoured in the early chilling; while sucrose was not; nevertheless, significant positive gradients before Bonferroni correction for sucrose contents indicated that, for plants in chilling treatments that flower early, selection favoured those that flowered with less quantity of reserve carbohydrates. In the non-chilling treatment where plants that flowered late with more leaves, selection favoured more reserve carbohydrate storage.

5.4.5 Detection of plasticity costs[1]

[1]Plastic cost is described as the reduction in fitness of plastic organisms compared with less plastic organisms in a given set of environment.

The costs of plasticity were estimated by standardized regression coefficients of a species' mean relative fitness on its plasticity term. We considered only the traits that were favoured in the selection analysis. We detected negative terms for flowering time, leaf number, allocation to leaf, SLA, LMR, and fructans (Table 4) under early chilling treatment. However, significant gradients were found for few traits suggesting that the species with greater plasticity for these traits had reduced fitness. Since these were plastic traits, these negative gradients were interpreted as a cost of

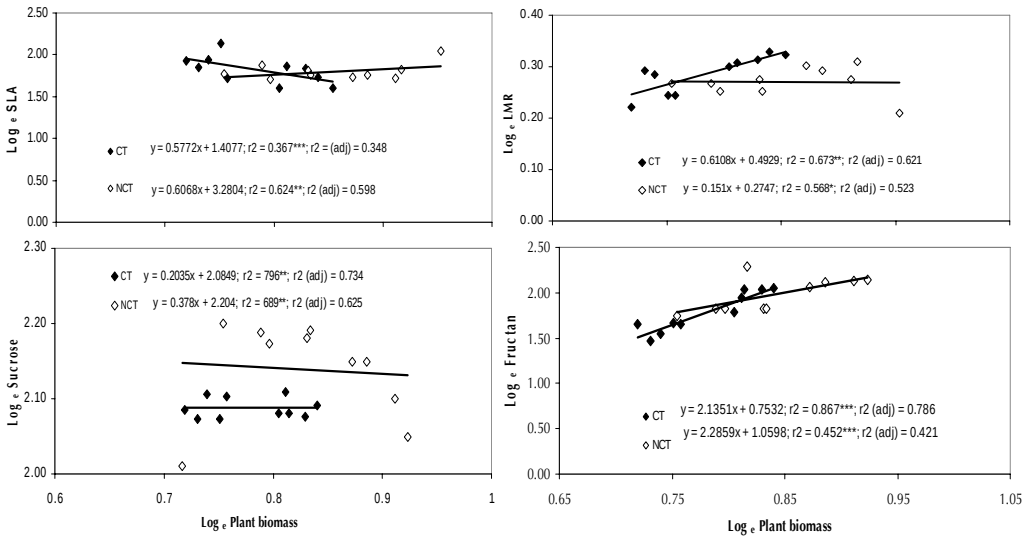

Figure 4 Linear regression analysis between plant biomass and specific leaf area (SLA), leaf mass ratio (LMR), sucrose and fructan for two Triticum species grew under chilling (CT) and non-chilling (NCT) temperature conditions. Regression equations along with adjusted coefficients of determination for each trait under treatments were provided.

plasticity. In the non-chilling treatment, these gradients were positive and were not-significant even before Bonferroni correction (Table 4).

5.5 Discussion

Plant morphology diverges as the plant grows and develops from germination to maturity. This morphological variation is an outcome of, in part or in full, variation in the developmental processes and developmental pathways. Distinguishing developmental plasticity (environmentally induced variation in the developmental pathway of a trait) from passive plasticity (variation in a trait as a consequence of environmentally induced variation in growth) might refine the theoretical models of the evolution of plasticity. Thus, incorporating a developmental approach in studies of phenotypic plasticity may reveal a better understanding of a plants ability to respond to its environment (Wright & McConnaughay 2002; Niinemets 2004; Moriuchi & Winn 2005).

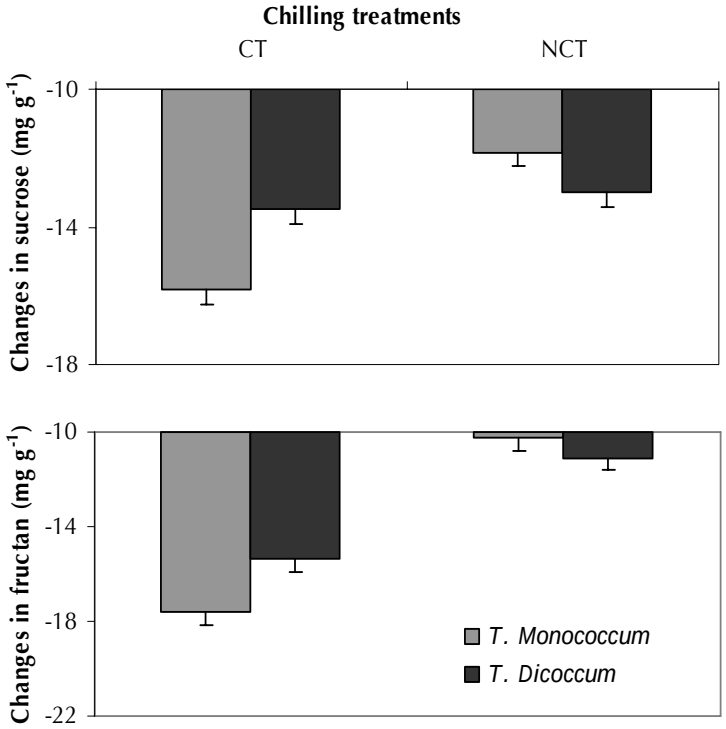

Figure 5 Changes in the concentrations of reserve carbohydrates in two *Triticum* species in response to two early seedling treatments. CT, chilling temperature; NCT, non-chilling temperature. Values are means ± SE.

Developmental plasticity studies on various stress gradients have provided valuable information leading to current understanding of the origin of (co)variation among morphological traits (Tomlinson & Anderson 1998; McConnaughay & Coleman 1999; Gunn *et al.* 1999; Harmens *et al.* 2000; Hopkins *et al.* 2008). Our study corroborates and further integrates these previous efforts in several ways. First, we found phenotypic plasticity that is not evenly expressed at two developmental stages, i.e. pre-flowering and flowering[1]. Second, selection analysis favoured earlier flowering under early chilling. Third, plasticity costs analyses suggested that more plastic species may have lower fitness under early chilling. Fourth, greater partitioning of biomass to foraging organs (leaves and roots) supports life-strategy theory and optimal partitioning models. These stress-induced (morphogenetic) responses are postulated to be part of a general acclimation strategy during the recovery period, but not to stunted growth, whereby plant growth is redirected to repress stress damage (Potters *et al.* 2007).

[1]In addition, plastic responses at pre-flowering can be overlooked when compared to plastic responses at flowering.

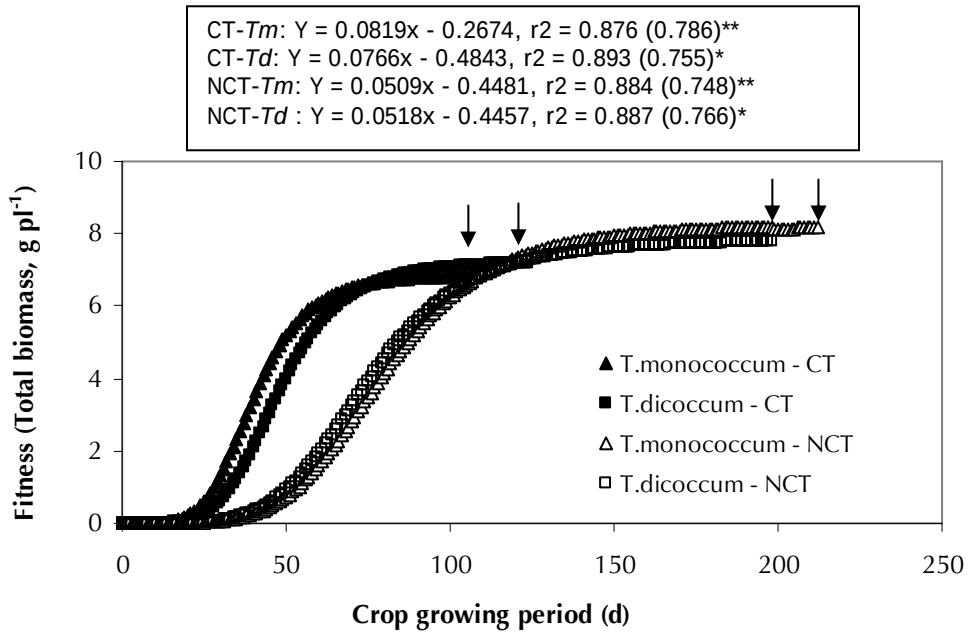

Figure 6 Regression of daily cumulative total plant biomass on crop growing period. Daily total biomass was derived using a simulation model (CERES-Wheat) for two species. In general, the magnitude of daily biomass accumulation represents growth curve (a typical sigmoidal curve) of a species. Given this perspective, a regression of total plant biomass on crop growing period revealed a different growth curve (developmental path) for early chilled plants that differed substantially to non-chilled plants. The arrows indicate flowering time of two *Triticum* species under two treatments. Regression equations along with adjusted coefficients are shown.

5.5.1 Ecological and adaptive significance of an early chilling

Previous studies have quantified selection on flowering time in many species. In a glasshouse study, direct selection favoured earlier bolting in *Arabidopsis* (Lee & Amasino 1995; Mitchell-Olds 1996). Consistently, in several field and potted studies, earlier flowering was favoured, sometimes in combination with selection favouring later bolting (Callahan & Pigliucci 2002). Callahan *et al.* (2005) documented strong selection favouring early bolting with smaller leaf number and lower plant mass to cold treatment. Our results are consistent with these previous efforts, as well as with the life-history theory that shows the selection may not favour both early flowering and larger plant mass simultaneously[1] (Stearns 1992; Callahan *et al.* 2005). Prolonged periods of cold, but not short episodes, may result in changes at multiple levels (e.g., chromatin remodeling) that down-regulate the expression of flowering locus C (*FLC*) (Aloia *et al.* 2008). The early flowering of plants could be interpreted as having ecological significance in their natural habitats. Indeed, several ecological studies have indicated that populations evolving from more stressful environments flower earlier (Aronson *et al.* 1992; Stanton *et al.* 2000).

[1]In addition, given the coordination between environmental factors and molecular signals, any environmental fluctuation requiring the adjustments in growth and development is a potential factor to regulate flowering.

Flowering time has been conceived to change the relations among morphological traits (Pigliucci & Schlichting 1998). Accordingly, flowering time, an index of length of growing period, is the trait on which mostly developmental decisions can be transmitted and implemented, including past stress signals (Kvaalen & Johnson 2007), where they may be subjected to natural selection (Schuermann *et al.* 2005; Bond & Finnegan 2007). We, therefore, propose that flowering time may also act as a signal trait that can be used to visualize developmental path for a phenotype. Considering a well-established 'growth curve' (a sigmoidal curve) perspective, a non-linear response between daily cumulative plant biomass (fitness) and crop growing period yielded a divergent developmental pathway for early chilled plants that differed substantially from non-chilled plants (Fig. 6). If plants develop along different developmental paths, it provides new insights into how different early environmental qualities affect the phenotype size and adaptive traits as they grow and develop (Moriuchi & Winn 2005). This different developmental pathway might be the major source of the morphological variation (Pigliucci & Schlichting 1998: Wilkins, 2002; de Kroon *et al.* 2005), which in turn influenced adaptive traits (Kvaalen & Johnson

2007). This developmental pathway is characterized with '*early flowering-smaller plant mass-smaller leaf size*' that may represent coordinated morphogenetic responses towards early chilling environment. Our results corroborate a previous study that found total plant size and architectural traits were ontogenetic determinants of fitness in a phenotypically plastic annual weed (*Amaranthus albus*) (Cheplick 2002).

Leaf traits are conceived to play an important role in adaptation to different environments. We observed early chilling temperature effects in flag leaf size (and length) as well as leaf mass ratio, but in opposite directions. Although leaf lengths were longer (data not shown), the proportion of total leaf length made up by leaf blade decreased (lower SLA). In contrast, leaf mass ratio (LMR) increased. Together, these results support a decrease in SLA (underpinned by increases in leaf thickness[1]) most likely because of the combined effect of decreased cell expansion and accumulation of reserve carbohydrates in response to early chilling environment (Nayyar *et al.* 2005; Singh *et al.* 2005; Kalberer *et al.* 2006; DeRidder & Crafts-Brandner 2008). Further, any increase in leaf thickness beyond threshold may also trigger feedback signal that potentially suppresses photosynthetic capacity[2] (Paul & Pellny 2003). Such smaller leaves typically have low photosynthetic activity, therefore carbon loss, and a long lifespan, which increases the 'revenue stream' per unit biomass invested in the construction of new leaf tissues (Westoby *et al.* 2000; Salgam *et al.* 2008; Hopkins *et al.* 2008).

[1]More leaf thickness is due to more number of cells present in a unit leaf area.

[2]This might occur in two ways: First, higher cell number per unit area results lower light penetration. Second, higher carbohydrate contents in the cell may suppress/reduce the net photosynthesis.

Although total selection for lower SLA to early chilling temperature was observed, we found no significant direct selection on this trait. This indicates that some of the total effect of SLA on fitness was indirect, i.e. mediated by other traits. One such candidate trait is relative growth rate (RGR), which was positively correlated with both SLA and fitness in early chilling environment. These results support the buffered roles of SLA on fitness, which explain the plasticity in SLA buffer fitness by contributing at least partly to homeostasis in vegetative growth rate (Shipley 2000; Steinger *et al.* 2003). These results are consistent with Villar *et al.* (2005) who reported the correlations between SLA and RGR were strongly influenced by ontogeny in wild *Triticum* species.

Early chilled plants showed a larger reduction in reserve carbohydrates suggesting that more reserve utilization in these plants. Reserve carbohydrates

may contribute significantly to a plant's fitness against harsh growing conditions (Kleijn *et al.* 2005). Previous studies have found that budburst (or flowering) in many temperate species depend on current photoassimilates (Eshghi & Tafazoli 2006; Sanz-Perez *et al.* 2008). If photosynthetic capacity is limiting (low current photoassimilates), it is likely that plants would depend on reserve carbohydrates for metabolic maintenance (Xue *et al.* 2008). The lower reserve carbohydrates in early chilled plants might thus support their involvement in long-distance signaling during floral induction and may accelerated the flowering process in early chilling plants (Roldan *et al.* 1999). Further, reserve carbohydrates supply immediate energy and carbon skeletons (Morvan-Bertrand *et al.* 2001), involved in stress tolerant mechanisms (Van den Ende et al., 2005; Valluru et al., 2008; Valluru & Van den Ende, 2008), acts as osmolytes (Kawakami *et al.*, 2008) and scavengers of reactive oxygen species protecting from chilling-induced oxidative stress (Van den Ende & Valluru, 2009). Hence, the utilization of stored reserves in early chilled plants seems to have dual strategic effects: on one hand, they support compensatory growth; on the other hand, they suppress stress damages. Empirical evidences suggest that if plasticity in functional traits (reserve carbohydrates in this case) enhances survival and fitness, this plasticity is considered adaptive (Pigliucci 2001; Griffith & Sultan 2005; Richards *et al.* 2006). Consistently, our selection analysis favoured lower fructan levels in response to an early chilling.

5.5.2 Plasticity costs to an early chilling

A cost of plasticity represents a reduction in fitness of a genotype as a consequence of expressing a certain phenotype through a plastic rather than fixed development. Five different plasticity costs can be found in the plasticity literature[1] (DeWitt et al., 1998; van Kleunen & Fischer, 2005; Valladares et al., 2007). Our study corroborates a few of these costs. First, costs for information acquisition are likely in our study background, as we measured plastic responses outside the treatment period. Although these costs are unlikely for plants, a series of recent stimulating studies strongly supports existence of 'stress memory or stress imprints', which predicts that plants can remember past stress and transmit stress signals (via epigenetic mechanism) to later stages and progenies, in some cases, up to several generations (Molinier *et al.* 2006; Galloway & Etterson 2007; Bond & Finnegan 2007; Kvaalen & Johnsen 2007; Bruce *et al.* 2007; Besnard *et*

[1]The five costs are: maintenance costs, production costs, information acquisition costs, genetic costs and developmental instability of environmentally contingent phenotypic features.

al. 2008). Second, the generation of smaller, but long-lived leaves by investing more biomass allocation per unit leaf area might impose construction or production costs. Although production costs in plants are a matter of debate (van Kleunen & Fischer, 2005), we argue if production costs can able to stimulate additional costs via functional traits, which involved directly in metabolic maintenance (maintenance costs), then the costs are likely to increase in early chilling (Richards *et al.* 2006).

The costs detected in the present study are in quantitative traits, which are environment-specific (*sensu* Sultan & Spencer 2002) and were significant for only a few traits after applying Bonferroni correction, a more stringent rejection criterion (Relyea 2002). Based on a less stringent rejection criterion, we can conclude that the detected costs are significant, yet, we found more plasticity costs for a life-history trait of large effect (flowering time) which is the basis for plasticity (Pilliucci & Schlichting 1998). Such a result is consistent with the variation between two *Triticum* species that we found for that trait which is controlled by multiple genes (Roux *et al.* 2006). If early chilling-mediated plasticity favours early flowering, then, why are late flowering populations of *Triticum* species so persistent in nature? Roux *et al.* (2006) postulated that during adaptive evolution, the purifying selection may have mostly been achieved via the fixation of a few mutations having large fitness effects, which confer the largest progress towards the optimal phenotype (late flowering in this case), while having the least negative pleiotropic[1] effects. In addition, these phenotypes may live longer; allocate more biomass to above-ground structures, and delay reproduction via scheduling later flowering allows plants to escape harsh winters and maximizes fitness. Further, they may also likely benefit from additional life-supporting mechanisms such as drought tolerance (Stinchcombe *et al.* 2004) and higher water use efficiency (McKay *et al.* 2003). Given these premises, the selection against early chilling may be counteracted by the selection to enhance life-supporting mechanisms.

[1]Pleiotropy occurs when a single gene influences multiple phenotypic traits. Consequently, a new mutation (changes in the nucleotide sequence) in the gene will affects all traits simultaneously.

5.5.3 Conclusions

In nature, depending on the ecological breadth, all plant species may be exposed to environmental stresses at some point of their life-cycle. The present

study demonstrates that exposure to an early chilling stress during the seedling stage of *Triticum* species resulted in adaptive responses at the flowering stage. These responses include early flowering, alterations in several morphological and functional traits. Further, these responses were strongly associated with a different selection pressure that leads to a divergent developmental pathway. These results emphasize consideration of a different developmental approach (developmental paths) in understanding early environmental stress limitations on the final size and development of the phenotype.

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5.5.4 References

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Chapter five described how early chilling affects total water-soluble carbohydrate storage during recovery period. It typically explained that WSC can be involved in metabolic maintenance when the current photoassimilates are deficit to support the plant metabolism. Although it is well accepted that WSCs or its components can able to protect plants from chilling and drought stress, the question remains to answer 'how'.

Chapter six will explains how fructan, a major carbohydrate portion of total WSCs, is transported from its site of synthesis (vacuole) to site of action (membranes). Such knowledge is essential for better understanding of protection mechanisms of fructan under abiotic stress.

Nothing happens unless first we dream. Indeed, every great advance in science has been elicited from a new audacity of the imagination.

Carl Sandburg & John Dewey

Chapter Six

FUTURE PERSPECTIVES

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Research Opinion

Freezing Tolerance by Vesicle-mediated Fructan Transport

Keywords

Abiotic stress, fructan, freezing tolerance, membrane stabilization, vesicle-mediated transport,

6.1 Abstract

Fructans are fructose-based polymers associated with freezing tolerance. They may act directly via membrane stabilization or indirectly either by (i) stimulating alternate cryoprotectants or (ii) interfering with cold/drought signaling. Fructans and fructan biosynthetic enzymes (FBEs), in general, are believed to be present in the vacuole. This paper, in particular, draws attention to the exceptional presence of fructans and fructan exohydrolase (FEH) activities in the apoplast of cold-stressed plants. This observation raises questions on the origin of apoplastic fructans and suggests that fructans are transported to the apoplast by post-synthesis mechanisms, perhaps induced by cold. We propose a conceptual vesicle-mediated transport model for the movement of vacuolar fructans to the apoplast, where they could assist in stabilizing the plasma membrane.

6.2 Introduction

Preserving a part of photosynthesized products in storage organs is a typical characteristic of many plants. The most prominent reserve carbohydrates are starch and sucrose. Fructans compose another form of storage carbohydrates occurring in about 15 % of the flowering species (Hendry, 1993). They act as (i) reserve carbohydrates to sustain grain filling and grass re-growth (Gebbing & Schnyder, 1999) (ii) protective agents against cold and drought stress through membrane stabilization (Hincha *et al.*, 2003; Hisano *et al.*, 2004) and (iii) as osmotic regulators during flower opening (Le Roy *et al.*, 2007). Additionally, inulin type fructans [linear β (2,1) linkages] have gained importance as functional food ingredients (Roberfroid, 2007)[1].

[1]In addition, fructans act as dietary fiber in the digestive system and selectively stimulate the growth of beneficial bacteria in the intestines. Further, they can also be used as sugar substitute and fat replacer. Commercially, fructans are produced from Chicory and Jerusalem artichoke.

Fructans are synthesized by fructan biosynthetic enzymes (FBEs). In the first step, the initiator enzyme 1-SST catalyzes the initial fructosyl transfer between two sucrose molecules. Further chain elongation occurs by other types of fructosyl transferases (1-FFT, 6G-FFT and 6-SFT) adding β (2,1) or β (2,6)-linked fructofuranosyl units. In this way, five types of fructans can be synthesized in plants (Van den Ende *et al.*, 2002). Fructan breakdown is accomplished by fructan exohydrolases (FEHs). Different types of FEHs (1-FEH, 6-FEH, 6-KEH and 6&1-FEH) have been recently described in fructan- and non-fructan plants (De Coninck *et al.*, 2007).

Fructan pool size is controlled by the balance between FBE and FEH activities. In dicots, 1-SST and 1-FEH are temporally separated (Van Laere & Van den Ende, 2002). However, in monocots FEHs are co-expressed with FBEs (Van den Ende *et al.*, 2003). Increasing evidence has been generated showing that fructans can be present in the apoplast, phloem and xylem tissues (Vieira & Figueiredo-Ribeiro, 1993; Livingston & Henson, 1998; Wang & Nobel, 1998; Ernst & Pfenning, 2000; Van den Ende *et al.*, 2000; Van den Ende *et al.*, 2005). Some monocot FEH enzymes are active in the apoplast (Livingston & Henson, 1998; Wang & Nobel, 1998; Ernst & Pfenning, 2000; Van den Ende *et al.*, 2000; Van den Ende *et al.*, 2005). Other reports support a vacuolar localization (Wagner & Wiemken, 1983), but FEH activity might be explained as a side activity of vacuolar invertases that are very prominent in monocot leaf tissues (Ji *et al.*, 2007). FBEs are localized in the vacuole, and were never detected in the apoplast (Van den Ende *et al.*, 2005).

Although many *in vitro* experiments convincingly demonstrated the protective nature of fructans in stabilizing membranes under stress, it remains unclear whether fructans directly protect membranes *in vivo*. One of the major problems is their putative vacuolar localization, preventing interaction with the plasma membrane. Here we summarize our knowledge on fructan metabolism and draw novel conceptual features for the most important, yet unsolved, problem in this research area: how could vacuolar fructans reach the plasma membrane? By considering these features, we propose a hypothetical vesicle-mediated model for the vacuolar fructan movement to the apoplast during cold hardening.

6.3 Fructans, FEHs and freezing mechanism

The plasma membrane is believed to be the primary site of freezing-injury (Steponkus, 1984). Both mono- and disaccharides are effective in membrane stabilization *in vitro*, while polysaccharides (hydroxyethyl starch, glucan and dextran) are not[1]. Fructans, however, behave differently and reduce the leakage of the soluble contents from liposomes, probably by depressing the transition temperature (t_m) at which the lipid phase changes from gel to liquid crystal (Hincha *et al.*, 2000). A study with inulins isolated from chicory roots and *Dahlia* tubers demonstrated that inulins stabilize egg phosphatidylcholine (PtdCho) during freeze-drying by maintaining a direct hydrogen bonding interaction between the phosphate groups of egg PtdCho and the inulin hydroxyls. Fructans behave more like low molecular mass sugars retaining additional water and reducing leakage from the liposomes after freeze-drying (Hincha *et al.*, 2000). This might be due to the greater mobility of the fructan chains with a -O-CH_2- between each constituent monosaccharide.

The breakdown of fructans is accomplished by fructan exohydrolases (FEHs). Apoplastic FEHs are proposed to depolymerize apoplastic fructans, and thus to generate a mixture of sugars (hexoses, sucrose and fructan oligosaccharides) that might be optimal for efficient membrane stabilization *in vivo* (Van den Ende *et al.*, 2005). Measurements of fructan protection of membranes *in vitro* showed that lower degree of polymerization[2] (DP) fractions (3-5) from cereals were more protective than higher DP fractions. Mixtures of fructans, as they occur in living cells, may have protective properties that differ significantly from those of the purified fractions (Hincha *et al.*, 2007).

[1] Monosaccharides are small in size so that they can easily be inserted in between lipid bilayers but polysaccharides are larger molecules, which sterically prevent them to be inserted between lipid bilayers.

[2] DP is the number of repeat units in an average polymer chain at time t, in a polymerization reaction. DP is used as measure of molecular weight.

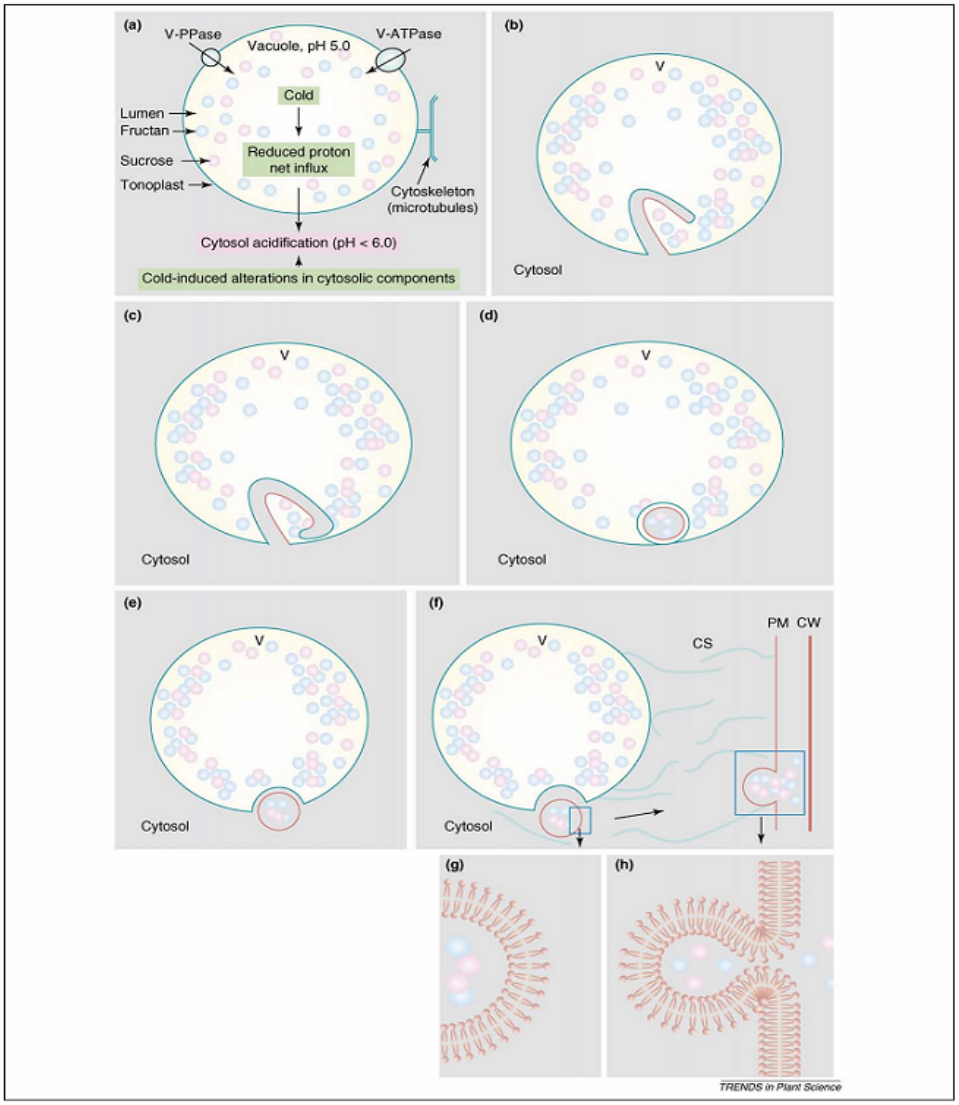

Figure 1 Possible mechanism of vacuolar membrane invagination. In the first step, A, cold induces inactivation of V-ATPase due to dissociation of its catalytic subunits. However, cold up-regulates V-PPase. Activation of V-PPase causes more pronounced effects on luminal pH. The differential pH gradient caused by cold might stimulate tonoplast deformation. B. Invagination of tonoplast. The deformed tonoplast might fold inward toward the lumen. C-D. The invaginated portion extends deeply into the lumen and may curve to connect to the donor surface, yielding nearly spherical double-membraned structures, and capturing vacuolar substances such as fructans and sucrose. E. The release of tonoplast vesicles into the cytosol. The double-membrane vesicles may uncoat at the tonoplast and released as single-membrane vesicles into the cytosol. F. The released single-membrane vesicles may move to the PM with the help of cytoskeleton structures and fuse with the membrane. After discharge, vesicles may integrate with the membrane (see inset, F1, F2 for magnified vesicle bilayer and its fusion with PM and releasing solutes). The released fructans may be selectively trimmed by apoplastic FEHs, and the resulting sugars may interact with PM lipids for membrane stabilization. V, vacuole; CS, cytoskeleton structures.

6.4 Vesicle-mediated mechanism and vacuolar solute transport

An apoplastic localization of fructans is at odds with the fact that FBE activity has never been detected in the apoplast. On the other hand, it is consistent with the presence of FEH activity in the apoplast. Based on these observations, we suggest that fructans might be transported from the vacuole to the apoplast through a vesicle-mediated mechanism[1] (VMM) that is distinct from the classical VMMs for secretory vesicles. In the latter, secretory vesicles move to the plasma membrane (PM) and vacuole via the endoplasmic reticulum (ER) and/or Golgi apparatus (GA). Two types of classical VMMs exist in multicellular organisms: constitutive (non-Ca^{2+} triggered) or non-constitutive (Ca^{2+}-triggered). One interesting feature of VMM in plants is its Ca^{2+} sensitivity (Battey *et al.*, 1999).

[1]VMM is a transport mechanism in which all substances are trapped in a round-shaped vesicle to transfer from one place to another place within the cell.

Over the last few years, it became clear that the endomembrane compartments are also involved in more specific processes in plants such as abiotic stress tolerance and pathogen defense. Abiotic stresses seem to up-regulate certain intracellular vesicle trafficking proteins that could possibly induce stress tolerance in plants (Mazel *et al.*, 2004). Although, little information is available on vesicle trafficking relative to environmental stresses, transgenic plants that constitutively express certain vesicle trafficking proteins (e.g. AtRabG3e) showed increased tolerance to osmotic stresses (Mazel *et al.*, 2004).

6.5 Vacuolar solute transport

Solute transport from the vacuole involves one or both of two pathways: carrier-mediated transport, probably for short distances, which refers to individual solute molecule movement across the tonoplast by facilitated diffusion or an energy-assisted mechanism (Echeverria, 2000); and vesicle-mediated transport, allowing large-scale movement of selectively captured compounds enclosed within small membranous vesicles (Echeverria, 2000; Etxeberria & Gonzalez, 2003). These two distinct transport mechanisms are not necessarily mutually exclusive, but might operate in parallel and/or in tandem to transport solutes from a vacuolar localization (Etxeberria, 2005).

Membrane and solute transfer from the vacuole is not unusual, as many vacuoles seem to employ tonoplast-vesicle-derived exocytosis (TVE) (Etxeberria & Gonzalez, 2000). Compelling lines of evidence stem from the observation of

long-distance transport of reserve substances (sucrose) in vesicle compartments (Echeverria & Achor, 1999). Although not yet fully proven, TVE is strongly suspected as a basic mechanism to carry vacuolar substances in plants (Yano *et al.*, 2004; Exteberria *et al.*, 2007) and has been demonstrated in yeast (Bryant *et al.*, 1998; Bowers & Stevens, 2005; Lee *et al.*, 2006). Recently, the vacuolar membrane material flow for the formation of autolysosomes has been demonstrated in tobacco cells (Yano *et al.*, 2004). Supporting this notion is the identification of a plant retromer complex[1] localized to the prevacuolar compartment and microvesicles in Arabidopsis (Oliviusson *et al.*, 2006). Most interestingly, direct endocytic vesicle trafficking from the PM to the vacuole is becoming increasingly apparent in plants (Etxeberria *et al.*, 2007; Etxeberria *et al.*, 2005; Etxeberria & Gonzalez, 2005), strongly supporting the probable existence of complementary mechanisms.

[1]A retromer complex is a protein complex that helps to sort solutes from the endosomal and /or prevacuolar compartments to the trans-Golgi network.

Environmental stresses, biotic or abiotic, induce significant intracellular remodelling in plants. The vacuole shows unusual architectural behaviour under diverse osmotica (Reisen *et al.*, 2005) and appears to disperse via progressive deformation and numerous folding events, suggestive of vesicle budding (Silady *et al.*, 2008). The presence of dispersed vacuoles is biologically consistent with several microscopic and fluorescence marker studies (Yamamoto *et al.*, 2003; Hicks *et al.*, 2004). In general, the vesicle budding machinery seems to be promoted by luminal acidification (Schumacher, 2006). Recently, it was shown that the activity of V-ATPase (which is a primary proton pump, located on the tonoplast that maintains cell pH homeostasis) is essential for sorting the solutes of endocytic and secretory trafficking in Arabidopsis (Dettmer *et al.*, 2006).

6.6 Conceptual model for vacuolar fructan transport

Previous studies suggest that luminal vesicle budding can arise via two mechanisms: autophagy[2] and/or invagination/evagination. Autophagy occurs during vacuolar degradation of cytoplasmic components and functions efficiently in young and non-senescence tissues (Slavikova *et al.*, 2005). The other mechanism involves membrane folding, either inward (invagination; [Saito *et al.*, 2002; Uemura *et al.*, 2002]) or outward (evagination; [Echeverria, 2000]). Invagination would probably yield small double-membrane vesicles,

[2]Autophagy is a degradation process. All dangerous cell substances may enter into vacuole and degraded into less dangerous compounds.

whereas evagination would produce single-membrane vesicles. These two processes might be specific to stress conditions (Saito *et al.*, 2002).

We suggest that inversion of the membrane might be stimulated under cold stress. When plants are exposed to cold, they rapidly inactivate their V-ATPase proton pump (Matsuura-Endo *et al.*, 1992) through disassociation of its catalytic subunits (Figure 1a). V-PPase acts as an auxiliary fail-safe pump, but the net proton influx decreases under cold stress, resulting in cytoplasmic acidification (Dietz *et al.*, 2001; Kawamura, 2008). The dissociation of catalytic subunits by abiotic stress might be one of the triggers stimulating tonoplast deformation and membrane invagination (Figure 1b). Studies of yeast demonstrated that mutants lacking vacuolar ATPase-V0 subunit Vph1p (Bayer *et al.*, 2003) and the Golgi or endosomal isoform Stv1p (Perzov *et al.*, 2002) showed fragmentation of the vacuole into multiple small vesicles. Conversely, the presence of Vps41-type V0 subunit (Takeda *et al.*, 2008) induced fragmentation too. Studies of plants and animals suggest that V-ATPase subunits directly interact with proteins regulating endocytic trafficking (Padmanaban *et al.*, 2004; Hurtado-Lorenzo, 2006). The activity of V-ATPase, decreased after initial exposure to cold, might be restored after prolonged cold exposure (Yoshida *et al.*, 1999). Changes associated with vacuole fragmentation and accumulation of vacuolar fructans might assist in stabilizing the inner side of the tonoplast membrane, which carries V0 subunits of V-ATPase. These mechanisms might allow water uptake to restore the osmotic balance of the cell (Baars *et al.*, 2007).

Deeply invaginated membranes might form double-membrane vesicles inside the vacuolar lumen (Figure 1b–d), capturing vacuolar compounds (including fructans and sucrose) during their formation. These vesicles might be uncoated at the tonoplast and released into the cytoplasm as single-membrane structures (Figure 1e,g) (Stefanowska *et al.*, 2002). Subsequently, they might be directed to the PM by components of the cytoskeleton and/or by tethering proteins. Finally, these vesicles fuse with the PM (Figure 1f) (Yamamoto *et al.*, 2003), mediated by certain trafficking proteins that might be incorporated during their formation. Fusion facilitates solute discharge and might include vesicle integration into the PM (Figure 1h). More likely, the extensive membrane loss at the vacuole under cold stress (Stefanowska *et al.*, 2002; Kawamura & Uemura, 2003) might be compensated by vesicles moving from the PM to the vacuole, a process that might be coordinated by intermediary multivesicular bodies[1] (MVBs). The

[1]MVB is a vesicle generated from plasma membrane and transports the solutes from plasma membrane to vacuole.

released fructans could be trimmed by apoplastic FEHs, and the resulting mixture of oligofructans and hexose sugars could interact with PM lipids for membrane stabilization (Van den Ende *et al.*, 2005). Moreover, the selective trimming of fructans by FEHs could assist in freezing point depression by lowering the osmotic potential in the apoplastic space.

6.7 Fructan–lipid interaction

In addition to their osmotic contribution after (partial) degradation, fructans directly interact with membrane lipids (Figure 2). Levan-type fructans preferentially interact with small-headgroup lipids. Inulin-type fructans show a deep interaction with lipids (Vereyken *et al.*, 2003). Compared to the helix conformation of levan-type fructans, the flexible, random-coil structures of inulin might insert much deeper into membranes, making a direct hydrogen bond with the phosphate groups (Hincha *et al.*, 2000). Inulin-type fructans can even interact with the C — O groups (Cacela & Hincha, 2006). Additionally, inulins showed a lower crystallization rate above the devitrification temperature[1] (Tg), so that, compared to trehalose, inulins provide better protection of membranes (Hinrichs *et al.*, 2001).

[1]Tg is the temperature at which a polymer becomes brittle on cooling or soft on heating.

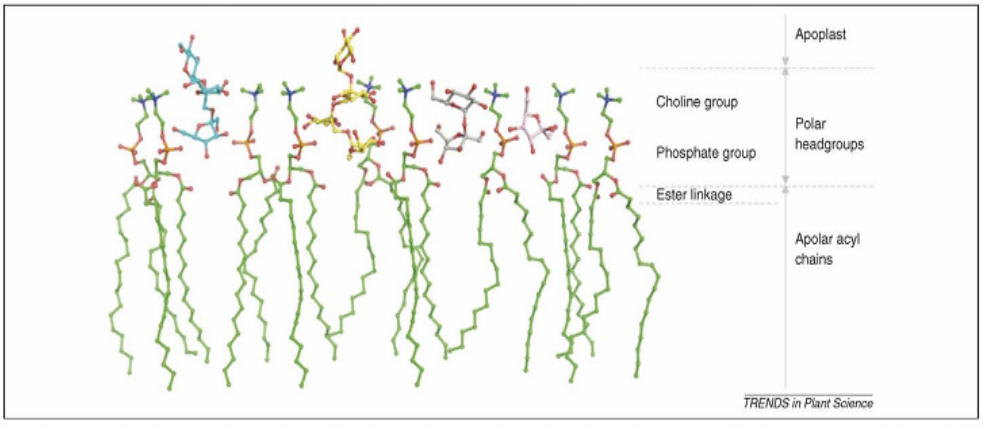

Figure 2 Simple representation (not docking) of 1-kestose (light blue); 1,1 nystose (yellow); sucrose (grey) and fructose (pink) inserted between the headgroups of phosphatidylcholine membrane lipids (phosphate: orange; carbon: green; nitrogen: blue; oxygen: red). Coordinates of 1,1 nystose and the membrane lipids were calculated using molecular dynamics (Timmermans *et al.*, 1993; Heller *et al.*, 1993). Picture was drawn with Pymol (Delano, 2002).

6.8 Relevance of the model

The proposed model of vesicle-mediated fructan transport from the vacuole to the PM is supported by several observations. First, the tiny vesicles budding off from the vacuole (after inward invagination of the tonoplast) might represent the mobile spherical structures frequently observed in the tonoplast of GFP-labelled tobacco leaves (Kotzer *et al.*, 2004), Arabidopsis cotyledons (Saito *et al.*, 2002), hypocotyls (Avila *et al.*, 2003), germinating pollen (Hicks *et al.*, 2004) and *Nicotiana benthamiana* plants (Escobar *et al.*, 2003). Most curiously, and perhaps even more convincingly, Klich *et al.* (2000) provided evidence of the total breakdown of the tonoplast into many small vesicles under PEG-mediated water stress.

Second, the mass transport of solutes through vesicles moving from the vacuole to the PM validates the recent point of view that VMM also involves alternative or parallel transport mechanisms in addition to the classic routes of protein transport within the cell and to the extracellular compartment. The bulk movement of solutes (fructans) facilitates rapid hydrolysis in the apoplast and rapid avoidance of cell damage by cold. Indeed, during spring regrowth, reserve (sucrose) mobilization seemed to occur more efficiently by a vesicle-mediated mechanism (Echeverria & Achor, 1999).

Third, cold-acclimated plants usually accumulate sucrose in their photosynthetic organs (reduced sucrose export but continuing photosynthesis), triggering fructan biosynthesis in these tissues. This sugar accumulation in the extravacuolar space (Livingston & Henson, 1998) might stimulate membrane folding. For example, guard cells of *Samanea* spp. exposed to a variety of environmental signals lose their ability to maintain their high vacuolar content; the process is accompanied by changes in vacuolar volume and shape, and the appearance of vesicles (MacRobbie, 1999). Indeed, vesicle budding from the central vacuole was proposed 50 years ago based on microscopic observations in *Mimosa pudica* under chilled conditions (Weintraub, 1951).

6.9 Conclusions and perspectives

Fructans have been detected outside cells, although they are synthesized within the cell. In particular, some fructans and FEHs reside in the apoplast of cold-

acclimated plants. We propose here that the bulk movement of solutes occurs through TVE, a process that might actually occur in many plant species. The proposed model enables efficient transport of fructans (and sucrose) from the vacuole to the PM. Apoplastic FEHs might selectively degrade these fructans, producing a cocktail of hexoses, sucrose and oligo-fructans, providing optimal membrane protection in vivo, as observed in vitro. Additionally, selective FEH trimming might result in freezing point depression by lowering the osmotic potential.

It is obvious, however, that freezing tolerance is a very complex process and direct membrane protection by fructans might only explain one part of a combined strategy. Additionally, it remains possible that fructans could protect tissues indirectly. Indeed, their water-soluble nature (in contrast to starch) allows, even at low temperature, rapid production of carbon and energy for the synthesis of alternative cryoprotectants (e.g. proteins). Accumulation of vacuolar fructan oligosaccharides lowers the osmotic potential, affecting water influx from the cytoplasm to the vacuole and putatively interfering with drought signalling, which is known to be cross-linked with cold signalling (Kawakami *et al.*, 2008). Whatever the mechanism(s) involved, recent investigations convincingly demonstrate that the introduction of 1-SST to the non-fructan-containing plants tobacco (Li *et al.*, 2007) and rice (Kawakami *et al.*, 2008) effectively contributes to freezing or chilling tolerance. It is now a challenging task to generate more conclusive evidence for the existence of fructan transport between the vacuole and the PM. This would constitute an important milestone on the road to a full understand of the multifaceted roles of fructans in *planta*.

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Before speaking, consider the intent as well as the interpretation of your words, as our virtues lie in the interpretation of the results.

Andrew Alden & William Shakespeare

Chapter Seven
GENERAL DISCUSSION

General Discussion

Wheat is one of the most important staple food crops of the world, occupying 17% (one sixth) of crop acreage worldwide, feeding about 40% (nearly half) of the world population and providing 20% (one fifth) of total food calories and protein in human nutrition (Gupta *et al.*, 2008). Wheat production during the last four decades had increased steadily, however, a fatigue has been witnessed during the last few years, leading to the lowest ever global wheat stocks since 1948/49. Furthermore, it is projected that, to keep face with growing human needs, wheat grain production must increase at an annual rate of 2%, without any additional land to become available for this crop (Gill *et al.*, 2004). To meet this evergreen challenge, a new level of understanding of the structure and function of the wheat plant is required.

In the last two decades, numerous research groups have made a significant progress in research areas that have high potential to boost wheat productivity through genetic interventions: First, by increasing the efficiency of carbon fixation[1] in C_3 species (Parry *et al.*, 2003, 2007; Long *et al.*, 2006; Zhu *et al.*, 2008). Second, a substantial body of work pointed to the pivotal role of spike fertility in determining yield potential (Fischer, 1985, 2007; Slafer & Savin, 1994; Reynolds *et al.*, 2005; Shearman *et al.*, 2005; Miralles & Slafer, 2007). Third, the development of the first comprehensive mechanistic model using physical parameters that cause lodging in wheat[2] (Berry *et al.*, 2007). Fourth, the genetic tools[3] that can take these physiological platforms closer to breeding application (Collins *et al.*, 2008). However, it was commonly, and to a large extent accepted, that the genetic gain in wheat yields during the 20th century can largely be explained by an increasing partitioning of biomass to reproductive organs (harvest index, HI), without major changes in biomass production (Brancourt-Hulmel *et al.*, 2003).

The present thesis is composed of four major chapters (3, 4, 5, and 6). **Chapter 3 described several morpho-physiological traits regulating WSCs under**

[1] A review of literature suggests that the average photosynthetic efficiency in C3 plants is only 4.6% at current atmospheric CO_2, but it is 6% for C4 species.

[2] According to this lodging-resistant model, however, one detrimental effect of breeding is the requirement of large dry matter to stems and roots. Also, lodging-resistant ideotype could be constrained by genetic linkage between the lodging-associated traits.

[3] As the crop performance is the end result of thousands of genes and their interactions with environmental factors and cultural practices, the most appropriate genetic tools that can be used for improving crop production are QTL-based approaches.

three N levels. Identifying such traits is important for better understanding of WSCs role in plant growth and development.

Stem biomass is a function of partitioning between stem structural dry matter (cell-wall polysaccharides) and stem WSCs. The reduced relative competition for limited resources for stem growth might increase spike growth i.e. spike index, which might improve grain set, grain number and grain yields (Austin *et al.*, 1980; Fischer, 1985; Demotes-Mainard & Jeuffroy, 2004). Unlike spike development, which starts relatively early in the developmental stages of floral initiation (Slafer & Rawson, 1994), spike growth takes place during a rather short window of phenological time starting about 20 d before ear emergence, in parallel with stem growth and elongation (Kirby, 1988). However, if the stem elongation phase is longer, which encompasses the spike growth period; it would enhance the accumulated crop growth during stem elongation period. This would result in a larger spike during this period, given that dry matter partitioning to the growing spikes does not change in response to the lengthened duration (Slafer *et al.*, 2005). This suggests that the possible extension of the stem elongation period, without changing the total crop length from sowing to anthesis, may raise grain number and further yield potential[1] (Slafer & Rawson, 1994; Whitechurch *et al.*, 2007).

[1]A potential adaptive explanation between grain number, yield and resource availability within the plant could be 'a plant could adjust the grain number to match the resource defined yield level'. This view explains that grain number "ordered up" matches the resource accumulation in the plant (Sinclair & Jamieson, 2006).

Nevertheless, the period of development of floral primordia (rapid spike growth), which partially overlaps with the period of rapid stem growth that acts as a sink for WSC accumulation leads to unhealthy competition. Thus, a special attention on the investment of WSC accumulation can be a potential alternative competitive sink for the spike growth, usually referred to as sink-sink (stem-spike) competition (Sadras & Denison, 2009), for the limited resources in the weeks before anthesis (Cruz-Aguado *et al.*, 1999; Foulkes *et al.*, 2007; Reynolds *et al.*, 2009). This would also seem logical given the physiological explanation for the successful use of dwarfed genotypes, which shifted resources captured towards the spike during the period of rapid spike growth (Brooking & Kirby, 1981; Fischer & Stockman, 1986). Moreover, Beed *et al.* (2007) found that stem WSC accumulation during stem elongation period is more sensitive to shading than spike or stem structural dry matter. This suggests that stem WSC accumulation would occur only if the sink demand of spike and stem structural biomass has been satisfied.

Another possible path for trade-off between grain number and WSCs is related to N. The N accumulation and partitioning to the spike during the period of rapid spike growth is positively correlated with grain number (Abbate *et al.*, 1995; van Herwaarden, 1995; Demotes-Maynard *et al.*, 1999; Demotes-Maynard

& Jeuffroy, 2004). Previous studies in which N supply is manipulated have found that the higher WSCs at anthesis were negatively correlated with protein concentration in above-ground biomass (van Herwaarden *et al.*, 1998). Thus, it is most likely that lower spike N could compromise grain number per spike in genotypes that accumulate more WSCs (Dreccer *et al.*, 2009).

Overall, the above information suggests that stem WSC accumulation is invariably influenced by simultaneously growing sinks. Given this compromise consequence, the identified morphological and physiological traits regulating stem WSC accumulation would help breeders to select a specific trait for improving WSCs.

A couple of field experiments with four genotypes under three N levels (see chapter 3 for more experimental details) to identify traits regulating stem WSCs. Our results suggest that in all N situations, higher WSCs are primarily associated with five measured traits such as vegetative biomass, flag-leaf width, root: shoot ratio, radiation use efficiency and N concentration in the plant. However, the regulation of each trait is closely and interactively influenced by other physiological traits such as chlorophyll content, net assimilation rate, and photosynthetic efficiency. However, the magnitude of each trait varied significantly depending on the N status, and the relations between these traits changed drastically. Here, three major vegetative traits and their regulation on total WSCs accumulation under different N levels were considered.

In the study, total V_{MASS} was negatively correlated with total WSCs contents. A higher V_{MASS} is not a good sign for high WSCs[1]. Higher V_{MASS} represent more stem structural biomass, which is mainly made up of cell-wall polysaccharides such as cellulose and hemicellulose. A partitioning of carbon to these cell-wall polysaccharides may potentially reduce the total WSC accumulation in the stems. Antuono *et al.* (1998) found a negative correlation between pyrolysis products of cellulose and hemicellulose and carbohydrate contents in two primitive species, *T. monococcum* and *T. dicoccum*. Consistently, Xue *et al.* (2008) found lower cellulose and hemicellulose contents and higher WSCs in several wheat lines. This observation is also consistent with the expression of transcript levels of the genes involved in cell-wall polysaccharide synthesis. As cellulose and hemicellulose contents vary widely among wheat genotypes (Lempereur *et al.*, 1997), it could be reasonable to speculate that total WSCs may also vary among wheat genotypes.

[1]High N level may synthesize more amino acids, develop more sinks, and improves sink capacity (spike growth) that consume more carbohydrates.

However, lower stem WSCs in the plants do not really represent higher partitioning of carbon to stem cell-wall polysaccharides. Indeed, developing spikes may also act as a competitive sink for carbon during the period of stem

elongation. Although, higher carbon partitioning to spike growth, instead of carbon partitioning to stem WSCs or cell-wall polysaccharides, might be associated with increased grain number (Reynolds *et al.*, 2009), it could finally end up with lower single grain weight because of lower partitioning of stem WSCs during grain-filling (Reynolds *et al.*, 2009).

When plants have higher N availability, they synthesize more amino-acids, develop more sinks and improve sink capacity to utilize more carbohydrates. This suggests that (1) high N increases more structural biomass, which is composed of more cell-wall polysaccharides, which results in lower stem WSCs; (2) improved sink capacity due to high N might utilize more carbohydrates, which also decrease total WSC storage. The carbohydrates could be used to fuel the synthesis of structural material, mostly in the developing leaves, which indeed act as strong proximal sinks for carbohydrates particularly during vegetative growth (Ainsworth *et al.*, 2007)[1].

[1]This interpretation is fairly supported by the fact that stems are the major reservoir for both nitrate (Magana *et al.*, 2009) and WSC storage (Gebbing, 2003).

When plants have low N availability, they synthesize less amino-acids, have lower sink development, and therefore reduced sink capacity to use carbohydrates. The plants grown under N-deficit stress, therefore, exhibit a decreased source: sink ratio, due to partial shedding of source leaves, as observed in this study. Indeed, plants may enhance the efficiency of N use at whole plant level and trigger more N absorption from the soil to maximize the whole-plant carbon gain[2] (Oikawa *et al.*, 2008).

[2]It has been estimated that, on average, leaves at the mean age of leaf shedding has sufficient net carbon surplus, on a 24-h basis, to support total respiratory costs roughly three times the night-time respiratory flux of the leaf itself (Reich *et al.*, 2009)

Another vegetative trait, FL_W fairly regulated WSCs. Higher FL_W increased WSCs due to higher flag-leaf RUE. In contrast, lower FL_W also increased RUE at whole-plant level. However, FL_W regulations varied widely depending on the N status of the plant. If plants had sufficient N supply, they increased FL_W, which eventually increased RUE, and therefore higher net photosynthesis (Shearman *et al.*, 2005; Izanloo *et al.*, 2008). This might increase carbon production and increase total WSCs. On the other hand, if plants were supplied with low N, FL_W decreased and resulted in lower flag-leaf RUE, but increased RUE at whole-plant level. Moreover, lower FL_W is normally associated with higher leaf thickness, which is an indicative of higher photosynthetic activity, also increased net carbon, and therefore more WSCs.

The RS_R maximized WSCs under low and medium N but not under high N level. Higher RS_R represents higher translocation of carbohydrates to roots, which may fairly decrease total WSCs. This may probably be related, at least partly, to closed or non-expanding canopies under low N levels (Cruz *et al.*, 2003)[1]. Conversely, a recent study found that N availability has little effect on the fraction of assimilates allocated for the production of a larger canopy, although

the fraction allocated to root system is greater, at low levels of N availability (Mäkelä *et al.*, 2008). This small effect on the canopy development has, in general, two effects: normal rate of leaf initiation and sustaining rates of existing sink leaves. Both effects contribute to decreased carbohydrate flux to the roots. Supporting this, most exponential-growth studies suggest that the carbohydrate flux to roots, regardless of the size of the plant, are explicitly regulated in order to meet the requirements of N, particularly under limited N availability (Mäkelä *et al.*, 2008).

Other traits that could fairly regulate WSCs were RUE and N_{LA}. RUE directly and positively regulated total WSC accumulation. However, N_{LA} was negatively correlated with total WSCs. This meant higher N status in the plant did not increase WSCs and more N availability may only extend N metabolism.

Given such different trait regulations, which varied widely under different N levels, simulating these responses might indeed pave a way to sound extrapolation of these results to other conditions. Considering this perspective, a simulation model for WSC accumulation under three N levels was developed and presented in Chapter 4.

Chapter 4 described a simulation model for WSC accumulation in wheat under three N levels. Physiological crop models are commonly used to answer agronomic questions. Since they typically explain the respective physiological processes at their downstream level, physiological crop models are a means to simulate the response of plants carrying diverse combination of alleles under different scenarios of environmental conditions. In the present study, the developed phenological model for WSC accumulation described carbon accumulation in the form of different carbohydrates, which is regulated and integrated with its driving factor, temperature. Although the model was fitted into an exponential model, it still exhibits a gap that needs to be filled by integrating more components. Indeed, a non-linear additive model, instead of an exponential model, might fit more precisely; nevertheless, it requires a large number of model parameters that need to be synthesized through extensive experimentation. However, here the objective was to develop a simple means to simulate WSC accumulation, which might pave a way for further model developments.

Here, as a general model assumption, the entire plant, irrespective of morphological architecture, was considered to be composed of three compounds (sugars, cell-wall compounds and other chemical substances), which were linearly related to total biomass. Then the rate of these compound

[1]Nonexpanding canopies under low N might be due to reduced cell-expansion, cell divisions and altered conductance of the plants (Dodd *et al.*, 2002), possibly through the interactions with a nitrate-cytokinin signaling pathway (Forde, 2002).

syntheses and how they were altered by crop management factors such as N was integrated. For this, carbon availability and thermal time was considered for model development.

The model predicted higher WSC accumulation at early stages and lower WSC accumulation at later stages of crop growth, which is also consistent with the rate of WSC accumulation. The model predicted that the rate of WSC accumulation was higher in the early stages of crop growth and lower in later stages. The model validation produced a RMSE of 6.58, suggesting that the predicted values are nearly in agreement with the observed data. However, overall, the predicted rate of WSC accumulation was 31% higher than the observed data. The genotype Biscay showed lower rate of WSC accumulation; while Tommi showed higher rate of WSC accumulation. Comparing the rates of WSC accumulation under different N levels revealed that the rate of WSC accumulation was higher under N_{100}, lower in N_0 and medium in N_{200} level. These rates were consistent with the observed total WSC accumulation under each respective N level. However, the proposed model predicted the rate of WSC accumulation nearly close to the observed data only in low and high N level, and deviated largely in medium N level.

The model simulated total WSC accumulation well with observations of all N levels. In all N levels, the model predicted higher WSC accumulation. However, at high N level, the model simulated lower WSCs at early stages and higher WSCs at later stages. Under conditions of different N supply, the total WSCs were 11% and 7% higher than the observed WSCs in low and medium N levels, respectively, whereas in high N level, the predicted WSCs were 12% lower than the observed WSCs. This suggests that the pre-anthesis N application had marked effects on total WSCs. The duration of accumulation of these WSCs was also significantly modified by N nutrition. Overall, WSC accumulation was overestimated by the proposed model.

The evaluation of the model with the predicted data set indicated that the prediction errors for the rate of WSC accumulation were more, and the RMSEs were mostly between 20-30% in all N treatments. For total WSCs accumulation, the prediction errors for the rate of WSC accumulation were less, and the RMSEs were mostly less than 20% in all N treatments. This suggests that, although the model could not predict the rate of WSC accumulation well, it could predict the WSC accumulation close to the observed WSC accumulation.

This difference in the model predictions might be due to factors that are not included in the model such as moisture levels, other environmental factors such as photoperiod and abiotic stresses. Importantly, winter wheat completes its life

cycle in two different seasons, winter and spring, the occurrence of winter hardiness and the transitions between these two seasons might also fairly affect total WSC accumulation. Because WSCs are closely related to a plant's fitness, they might be used for buffering the competition with neighboring plants. How such environmental stresses, occurring either at early stages or later stages, can influence total WSC accumulation in wheat plants might be an interesting research perspective, and has been described in Chapter 5.

Chapter 5 described the phenotypic plasticity of wheat and WSCs in response to early chilling stress in two primitive species. For this study two primitive winter species, *Triticum monococcum* and *T. dicoccum* were used as they have different morphology, *T. monococcum* (an einkorn diploid species with AA genome, 2n=14) has strong glumes, narrow leaves and larger size, while *T. dicoccum* (an emmer tetraploid species derived from hybridization of two diploid wild grasses with AABB genome, 2n=28) has short internodes, wider leaf blades and smaller size. Also, they differ in yield, growth habit and winter hardiness, soluble carbohydrates and protein contents (Hidalgo & Brandolini, 2008).

Many plants normally face cold stress in temperate regions, and the plants ability (or lack thereof) to acquire cold tolerance is an important factor in their ecological and evolutionary dynamics. The consequences of fitness, benefits, and costs of cold stress are likely to be determined, in part, by whether plant (traits) responses are inducible (expressed only in response to specific stress) or constitutively expressed (Jackson *et al.* 2004). For example, adaptation to latitudinal cold gradients favoured smaller leaves, reduced leaf blade, and petiole lengths in *Arabidopsis* (Hopkins *et al.* 2008). These studies suggest that the plastic costs to cold gradients could originate either from genetic[1] or maintenance costs[2] or both, and were adaptive in nature (Karpilova *et al.* 1980). Since early chilling showed season-long negative effects on growth and development (DeRidder & Crafts-Brandner 2008), understanding how it generates phenotypic variation via influencing the developmental path of a phenotype is essential to estimate the variation in plant adaptation across cold environmental gradients and to predict the effects of impending climate change on plant production.

Chilling temperature led to earlier flowering in both *Triticum* species. Flowering occurred as early as 109 d and as late as 124 d following the chilling treatment, and the greatest delay in flowering occurred in non-chilling treatment. The two species differed significantly in flowering time. The differences in days to flowering between treatments were higher for *T.*

[1]Genetic costs are due to the pleiotropic effects of up-regulated FLC and FRI genes that are known to govern flowering time.

[2]Maintenance costs are due to the energy required for transcription and expression of FLC and downstream machinery, which must be maintained in the growing plant tissues after cold exposure.

monococcum that flowered 15 d earlier than *T. dicoccum*. Consistent with the size of both species, early chilling stress reduced whole-plant biomass significantly compared to non-chilling control. On average, the decrease in total plant biomass for early chilled plants was 18 and 24% at pre-flowering and flowering compared to control plants. On average, both species grown under early chilling conditions allocated 18 and 13% more biomass to leaf and root tissue, respectively. This resulted in higher leaf mass ratio (*c.* 27%) and decreased specific leaf area (*c.* 24%) with chilling treatment as plants grew larger. This indicates that, early chilling affected the allocation and leaf properties of both species. Furthermore, early chilling plants showed accelerated relative growth rate (*c.* 21%) than that of non-early chilling plants. Interestingly, when the total cumulative plant biomass was regressed on the crop growing period, early chilled plants showed a different developmental pathway. This different developmental pathway is associated with early flowering and lower plant biomass. Moreover, early chilling plants were *c.* 31% deficit in reserve carbohydrates.

A significant total selection (selection differential) favoured earlier flowering in early chilling conditions. Selection gradients estimated for flowering trait also indicated that flowering in fewer days with less leaf number and size was favoured in response to early chilling. Furthermore, more allocation to leaf, higher LMA, and lower SLA were favoured under early chilling. Plasticity costs were detected for flowering time, allocation to leaf, SLA, LMR, and fructans. Moreover, determinant analysis also suggested that these traits differed between the two species.

Combined, these results suggested that early chilling stress triggered variation not only in morphological architecture but also in functional traits such as reserve carbohydrates. Early chilled plants showed a larger reduction in reserve carbohydrates suggesting more reserve utilization, probably for the accelerated relative growth rate in these plants. Reserve carbohydrates may contribute significantly to a plant's fitness against harsh growing conditions (Kleijn *et al.* 2005). Previous studies have found that budburst (or flowering) in many temperate species depended on current photoassimilates (Sanz-Perez *et al.* 2008). If photosynthetic capacity was limiting (low current photoassimilates), it was most likely that plants would depend on reserve carbohydrates for metabolic maintenance (Xue *et al.* 2008). The lower reserve carbohydrates in early chilled plants might, thus, support their involvement in long-distance signaling during floral induction and may accelerate the flowering process in early chilling plants. Further, reserve carbohydrates supply immediate energy and carbon skeletons (Morvan-Bertrand *et al.* 2001), involved in stress tolerant mechanisms

(Van den Ende *et al.*, 2005; Valluru *et al.*, 2008; Valluru & Van den Ende, 2008), acts as osmolytes (Kawakami *et al.*, 2008) and scavengers of reactive oxygen species protecting from chilling-induced oxidative stress (Van den Ende & Valluru, 2009). Hence, the utilization of stored reserves in early chilled plants seems to have dual strategic effects: on one hand, they support compensatory growth; on the other hand, they suppress stress damages. Empirical evidences suggest that if plasticity in functional traits (reserve carbohydrates in this case) enhances survival and fitness, this plasticity is considered adaptive (Richards *et al.,* 2006). Consistently, our selection analysis favoured lower fructan levels in response to an early chilling. Further, discriminant analysis[1] also supported that allocation to leaf, SLA, flowering time and fructans were the most likely determinants responsible for the differences between two species.

[1]The first two axis in discriminant analysis explained about 72% to 81% of total variation between two species.

Untill now, I have described a small part of a vast unsolved research area, which helps in understanding WSCs and its functional roles in plants. Moreover, there seems to be unlimited potential for WSCs or its compounds that need to be identified. **Chapter 6 proposed an opinion for fructan transportation from the vacuole to the apoplast during chilling stress might occur through vesicle-mediated mechanism.**

This perspective describes 'freezing tolerance by vesicle-mediated fructan transport'. Thus, the proposed molecular model explains how fructan molecules could be transported from the site of synthesis (vacuole) to the site of action (membranes). The basic question behind this hypothesis was that enzymes responsible for fructan synthesis are absent in the vicinity of membranes (particularly in the apoplast), but fructans are found under chilling conditions. Thus, the question raised: how do fructans reach the apoplast? Here, I proposed a model for fructan transport through vesicles that originated from the vacuoles, induced by chilling stress, and vesicle-mediated transport of vacuole-based fructans to the membranes. After reaching the apoplast, transportation, fructans may depolymerise into hexose sugars and sucrose and participate in membrane protection under chilling stress.

Don't fear failure so much that you refuse to try new things. The saddest summary of a life holds three descriptions: could have, might have, and should have.

Louis E Boone

Chapter Seven

SUMMARY

Summary

Wheat is one of the major staple food crops of the world. Although a wealth of research has been made a significant progress in wheat productivity through genetic interventions in the last two decades, there remains an untapped potential for further yield gain. Water-soluble carbohydrates (WSCs) are excess carbohydrates stored in vegetative organs such as stem, sheaths, and tiller base during vegetative period. They are highly heritable agronomic trait that regulates plant growth and development as well as grain yields. In addition, WSCs also contribute to plant adaptation to abiotic stresses. Improving current understanding of the multi-faceted roles of WSCs is therefore essential for future crop improvement. The present thesis provides information on WSCs, its associated traits and future perspectives that derived from several experiments conducted under field and glasshouse conditions. Typically, the thesis has four objectives dealing with a specific set of questions.

The first objective explains the traits regulating WSCs under three N levels (0, 100 and 200 kg ha^{-1}). N concentration in the plant is negatively correlated with WSCs storage. The traits associated with total WSCs storage are also influenced by N levels. Three vegetative traits, *viz.,* total biomass, flag-leaf width, root: shoot ratio and two physiological traits, *viz.*, radiation use efficiency, and leaf N concentration were considered. Under high N level, lower biomass, flag-leaf width and root: shoot ratio is beneficial to increase total WSC storage. In contrast, increasing biomass and flag-leaf width is advantageous under lower N level. However, a specific set of traits, rather than a single trait, appeared to evolve under N-specific selection maximizing total WSC storage.

The second objective describes the simulation model for WSC accumulation under three N levels. A simple phenological model for carbon accumulation, in the form of WSCs, during vegetative period in four wheat genotypes was developed. This model was integrated and evaluated under crop management factors such as low (0 kg ha^{-1}), medium (100 kg ha^{-1}), and high (200 kg ha^{-1}) nitrogen supply. The proposed model predicted higher rate of WSC accumulation in the early stages of crop growth and lower rates in the later stages. Overall, the model predicted the rate of WSC accumulation with a RMSE of 6.58, suggesting that the proposed model simulated well. Nevertheless, the predicted rate of WSC accumulation was close to the

observed data only in low and high N level. The model predicted total WSCs well with the observed data; however, it overestimated total WSCs at early stages and underestimated total WSCs at later stages, largely due to the respective rate of WSC accumulation. Overall, evaluation of the model with the predicted dataset indicated that the prediction errors for the rate of WSC accumulation were more with RMSE between 20–30% in all N levels. For total WSC accumulation, the prediction errors were less, and the RMSE, in most cases, was less than 20% in all N levels.

The third objective reveals the plasticity of the phenotypic expression of two primitive wheat species (*Triticum monococcum* L. and *T. dicoccum* S.) in response to early chilling stress (4 °C). Early chilling stress resulted in lower total WSCs, in addition to lower flag leaf size, total biomass, specific leaf area and early flowering. While lower specific leaf area may reduce the early chilling stress effects at an individual leaf level, a higher leaf mass ratio and utilization of reserve carbohydrates indicated that the compensatory growth of chilled plants during the recovery period relied on the concerted action of altered resource allocations and reserve carbohydrate consumption. However, the lack of direct selection on sucrose indicates that sucrose has indirect effects on total WSCs. Thus, the total effects of reserve sucrose on relative fitness seem to be buffered via rapid growth rate in chilled plants. Nevertheless, a significant cost of plasticity was evident only for fructans. Further, a regression of daily cumulative plant biomass derived from a crop growth simulation model (CERES-Wheat) on crop growing period revealed a divergent developmental pathway for early chilled plants. These results showed that not only are the characteristic architectures in two *Triticum* species plastic, but the regulating mechanism of intrinsic developmental (ontogenetic) pathway is also sensitive to early chilling stress.

Fourth objective provides future perspectives for WSCs, in particular fructans. Fructans can be involved in freezing tolerance by protecting cellular membranes. This opinion postulates that fructans can be transported from vacuole (site of synthesis) to apoplast (site of action) through vesicles derived from the vacuole.

These results can improve the current understanding of WSCs in plant growth and development as well as grain yields. Traits can be used as WSCs markers to prescreen a large number of wheat germplasm for high total WSCs contents. However, a further understanding of different dimensions of WSCs in grain yield improvement and plant growth and development deserves more attention.

Zusammenfassung

Weizen ist eines der wichtigsten Grundnahrungsmittel in der Welt. Obwohl in den letzten zwei Jahrzehnten eine Fülle wissenschaftlicher Forschung zu einem signifikanten Fortschritt in der Weizenproduktion durch genetische Verbesserungen geführt hat, gibt es noch immer ein ungenutztes Potential für weitere Ertragssteigerungen. Wasserlösliche Kohlenhydrate (WLK, Saccharose, -Fruktane, Glukose und Fruktose) werden während des vegetativen Wachstums in Pflanzenteilen wie Stängelbasis oder Blattscheide gespeichert und sind hochgradig heritable agronomische Eigenschaften, die sowohl das Pflanzenwachstum und die Entwicklung als auch den Kornertrag beeinflussen. Weiterhin tragen sie zur zur Anpassung von Pflanzen an abiotischen Stress bei. Um die zukünftige Pflanzenproduktion zu verbessern, ist es notwendig, dass das derzeitige Verständnis für die Vielzahl von Steuerungsmechanismen von WLK verbessert wird. Die vorliegende Arbeit liefert Informationen über WLK und ihre Eigenschaften sowie daraus resultierende Perspektiven für die Zukunft anhand von Ergebnissen aus Feld- und Gewächshausversuchen Die Arbeit verfolgt vier Ziele mit unterschiedlichen, spezifischen Fragestellungen.

Das erste Ziel der Arbeit erläutert die Merkmale, die WLK unter drei unterschiedlichen N-Versorgungsstufen (0, 100 und 200 kg ha^{-1}) steuern. Die N-Konzentration in der Gesamtpflanze ist negativ mit der Einlagerung von WLK korreliert. Die Merkmale, die in die Gesamteinlagerung von WLK involviert sind, werden ebenso durch die N-Versorgungsstufen beeinflusst. In dieser Arbeit wurden drei vegetative Merkmale (Gesamtbiomasse, Fahnenblattbreite und Wurzel:Sproß-Verhältnis) und zwei physiologische Merkmale (Strahlungsnutzungseffizienz, Stickstoffkonzentration im Blatt) betrachtet. Bei hoher N-Versorgung scheint eine geringere Biomasse, eine geringe Breite des Fahnenblattes und ein geringeres Wurzel/Spross-Verhältnis die Einlagerung von WLK zu steigern. Dem gegenüber ist eine höhere Biomasse und eine größere Fahnenblattbreite von Vorteil unter geringer N-Versorgung. Vor allem die Kombination verschiedener Merkmale, und nicht einzelne Merkmale, schienen unter dem statistischen Ansatz einer N-spezifischer Selektion eine maximale Speicherung von WLK zur Folge zu haben.

Das zweite Ziel der Arbeit umfasst die Ableitung eines Simulationsmodells für die Akkumulation von WLK unter drei unterschiedlichen N-Versorgungs-

-stufen. In diesem Zusammenhang wurde ein simples (phänologisches) Modell für die Anreicherung von Kohlenhydraten in Form von WLK in der Pflanze während der vegetativen Periode von vier unterschiedlichen Weizen-Genotypen entwickelt. Das Modell wurde hinsichtlich des Managementfaktors N (geringe Versorgung (0 kg ha^{-1}), mittlere Versorgung (100 kg ha^{-1}) und hohe Versorgung (200 kg ha^{-1})) integriert und evaluiert. Das aufgestellte Modell überschätzte die WLK-Akkumulation in frühen Stadien des Pflanzenwachstums und unterschätzte die WLK-Akkumulation in späten Stadien. Zusammenfassend lässt sich sagen, dass das Modell die Rate der WLK Akkumulation mit einem root mean square error (RMSE) von 6.58 gut abbildete. Dennoch lag die über das Modell geschätzte Akkumulationsrate für WLK im Bereich l der geringen und der hohen N Versorgung nahe an den gemessenen Werten, wobei die größeren Abweichungen im mittleren N-Versorgungsbereich zu finden waren. Das Modell lieferte gute Vorhersagen für die Gesamtgehalte an WLK für die gemessenen Daten, doch überschätzte es die Gesamtgehalte an WLK in frühen und späten Entwicklungsstadien deutlich aufgrund der jeweiligen Rate für die Akkumulation von WLK. Unter verschiedenen N-Versorgungsstufen war der Gesamtgehalt an WLK 11 % bzw. 17 % höher als die gemessenen Werte bei geringer und mittlerer N Versorgungsstufe. Demgegenüber wurde bei der hohen N-Versorgungsstufe vom Modell ein 12 % geringerer Gehalt an WLK geschätzt. Die Gesamtbewertung des Modells für den genutzten Datensatz zeigte, dass die Schätzfehler für die Rate der WLK-Akkumulation größer waren, und dass der RMSE für alle N-Versorgungsstufen bei 20–30 % lag. Die Schätzfehler für die Gesamtakkukumulation der WLK war dagegen geringer und der RMSE lag für alle N-Versorgungsstufen bei etwa 20 %.

Das dritte Ziel der Arbeit war die Anpassung zweier ursprünglicher Weizenarten (*Triticum monococcum* L. und *T. dicoccum* S.) hinsichtlich der phänotypischen Erscheinung in Abhängigkeit von frühem Kältestress (4 °C). Ein früher Kältestress resultierte in geringeren Gehalten an WLK, und zusätzlich in einem kleineren Fahnenblatt, einer geringerer Gesamtbiomasse, geringerer spezifischer Blattfläche und einer früheren Blüte der Pflanzen. Während die reduzierte Spezifische Blattfläche die Effekte eines frühen Kältestresses auf dem Level einzelner Blätter reduzieren könnte, zeigten das höhereBlatt/Biomasse-Verhältnis und die Ausnutzung von Reservekohlenhydraten, dass das Kompensationswachstum von kältegestressten Pflanzen in der Erholungsphase auf die gezielte Veränderung der Ressourcenallokation und des Verbrauchs von Reservekohlenhydraten zurückzuführen war. In Ermangelung einer direkten Selektion auf Saccharose wird jedoch deutlich, dass die Saccharose einen

indirekten Effekt auf den Gesamtgehalt an WLK hat. Demnach scheint der Gesamteffekt der Reservesaccharosen auf die relative Fitness durch eine schnellere Wachstumsrate der kältegestressten Pflanzen gepuffert zu werden. Dennoch war ein signifikanter Aufwand an Anpassungsfähigkeit nur für Fruktane nachweisbar. Ein Rückgang der täglich aufsummierten Pflanzenbiomasse, abgeleitet vom Pflanzenwachstumsmodell CERES-Wheat, auf die Wachstumsperiode ergaben einen abweichenden Entwicklungspfad für kältegestresste Pflanzen. Diese Ergebnisse zeigen, dass nicht nur die charakteristische Architektur der beiden *Triticum* Spezies anpassungsfähig ist, sondern dass auch Regulationsmechanismen der spezifisch Entwicklungspfade auf Kältestress in frühen Entwicklungsstadien reagieren.

Das vierte Ziel der Arbeit zeigt die Zukunftsperspektiven für WLK, im Speziellen für Fruktane. Fruktane können in die Ausbildung von Kältetoleranz durch den Schutz der zellulären Membranen einbezogen sein. Der Autor kommt zu dem Schluss, dass die Fruktane von der Vakuole (Syntheseort) zum Apoplasten (Aktionsort) durch Bläschen aus der Vakuole transportiert werden.

Die Ergebnisse dieser Arbeit können das Verständnis von WLK im Pflanzenwachstum und der Entwicklung verbessern und genutzt werden, den Kornertrag zu steigern. Die untersuchten Merkmale können als WLK-Marker genutzt werden, um eine große Anzahl von Weizengenotypen auf hohen Gehalt an WLK zu screenen. Insgesamt sollte einem vertieften Verständnis der unterschiedlichen Dimensionen von WLK hinsichtlich der Ertragssteigerung und des Pflanzenwachstums und der Entwicklung mehr Aufmerksamkeit geschenkt werden.

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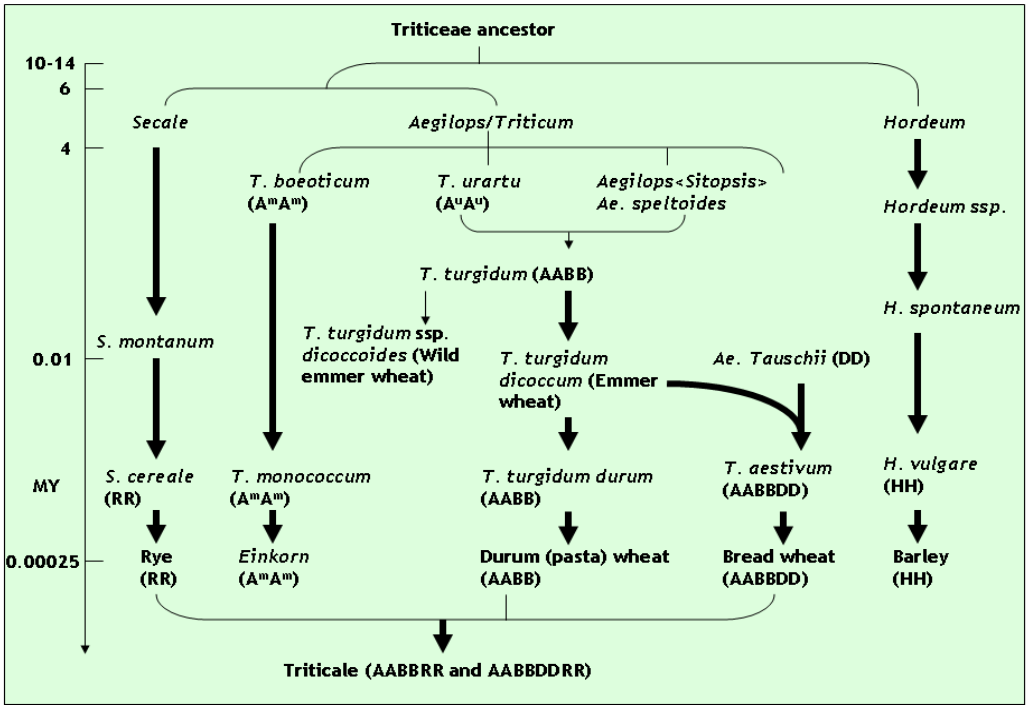
Appendix 1

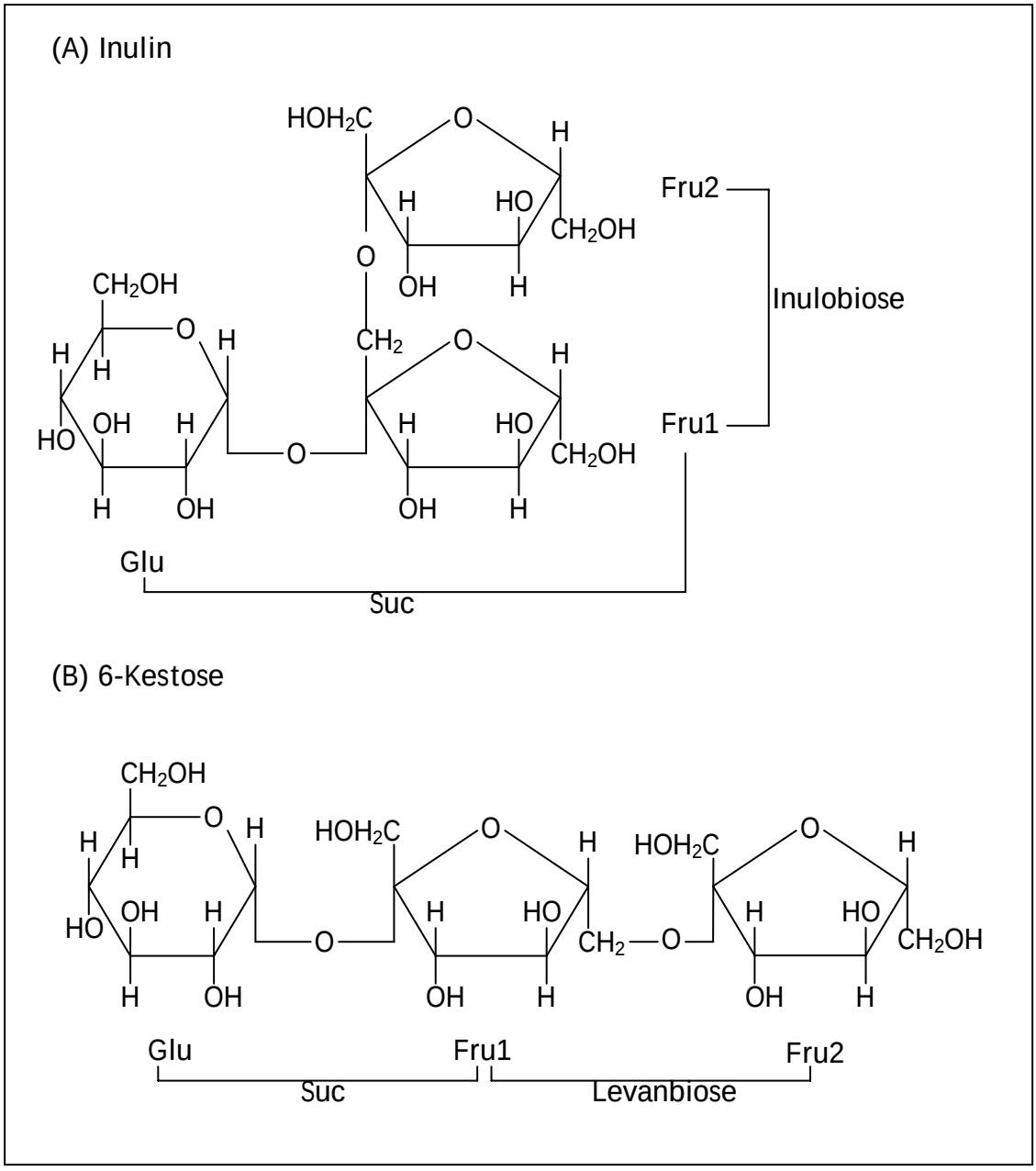
(Feuillet *et al.*, 2007)

Appendix 2



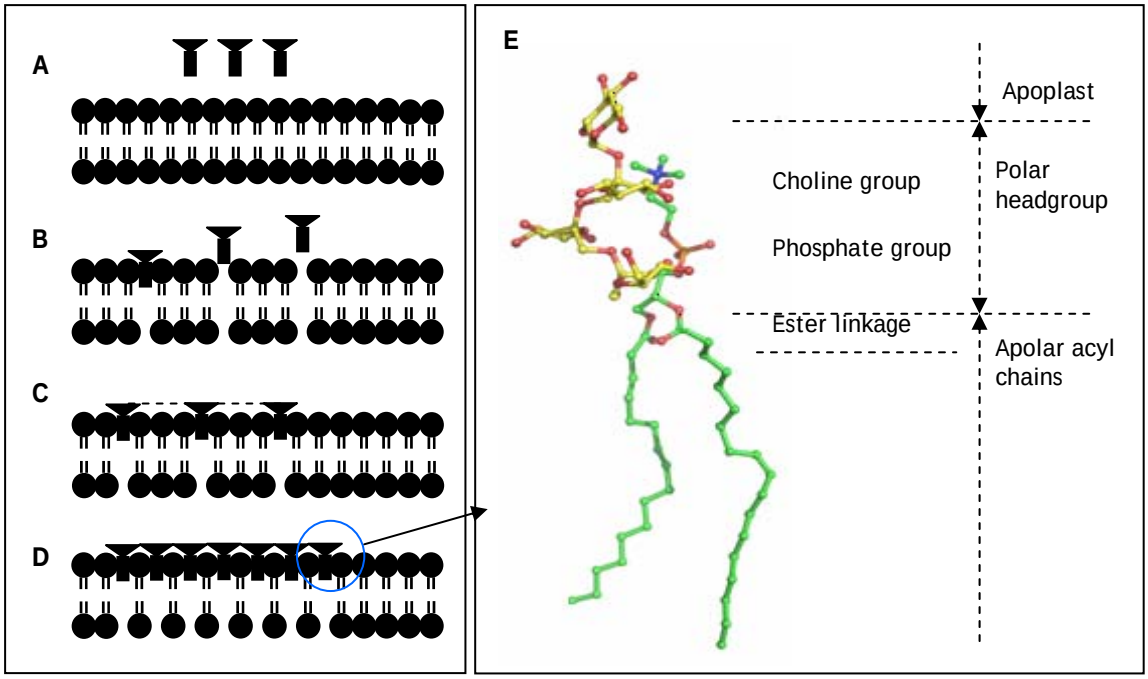
(Feuillet *et al.*, 2007)

Appendix 3



(Valluru & Van den Ende, 2008)

Appendix 4



(Valluru & Van den Ende, 2008)

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Educational background

PhD – Majored in Agronomy and Crop Physiology, University of Hohenheim, Stuttgart, Germany, 2007-09.
M.Sc – Majored in Agronomy, ANGRAU University, Andhra Pradesh, India, 1999-2002.
B.Sc – Majored in Agriculture, ANGRAU University, Andhra Pradesh, India, 1995-1999.

Academic achievements

Awarded a 'PhD Scholarship' from University of Hohenheim, Germany.
Conference travel grant from Federation of European Biochemical Societies (FEBS), UK.
Conference travel grant from New Phytologist Trust, UK.
National Eligibility Test (NET) from Agricultural Scientist Recruitment Board (ASRB), India.

Research experience

Research Technician at ICRISAT, India, March 2005 - April 2006.
Area Project Officer at AMEF, India, August - December 2004.
Senior Research Fellow at CRIDA, India, January 2003 - July 2004.
Research Associate at ARS, India, January - August 2002.
Research Executive in ITC Ltd, ILTD, India, August - December 2001.

Memberships

Member of Gesellschaft für Biochemie und Molekularbiologie e.V. (GBM), Germany (membership # 16249)

Research publications

Research publications – 9
Research reviews – 1
Research opinions – 2
General Scientific articles – 7
Proceedings – 1
Workshops – 1
Conference abstracts – 6

Others

Hobbies - Painting and Drawing

Personal details

Married - Dr. P.V. Bharathy.
Children - 1 daughter, Harshini Riya.

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