UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE
FACULTY OF AGRICULTURE
BIOTECHNOLOGICAL CENTRE

THE APPLICATION OF MOLECULAR TECHNIQUES IN PLANT BREEDING

Ph.D. Thesis

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České Budějovice
September 2011

This dissertation was approved for the degree of “Doctor of Philosophy” in
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Czech Republic
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Declaration

I hereby declare that the thesis entitled **"The application of molecular techniques in plant breeding"** has been carried out in the Biotechnological Centre, Faculty of Agriculture, University of South Bohemia in České Budějovice, Czech Republic under the guidance of Professor Vladislav Čurn, and in CRA-Genomics Research Centre, Fiorenzuola d'Arda, Italy under the guidance of Director Dr. Luigi Cattivelli. 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 acnowledged in the thesis.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AAO	Abscisic aldehyde oxidase
ABA	Abscisic acid
ABRE	ABA responsive element
AREB/ABF	ABA-reponsive transcription factors
ATHK1	A transmembrane histidine kinase receptor
CAMTA	Calmodulin binding transcription activator
CaMs	calmodulins
CAPS	Cleaved amplified polymorphic sequences
CM	Conserved motif
CMS	Cytoplasmic male sterility
CBL	Calcineurin B-like proteins
CDPKs	Ca^{2+} dependent protein kinases
C_t	Threshold cycle
CV	Coefficient of variation
DRE/CRT	Dehydration responsive element with C-RepeaT consensus
ERD1	Early response to dehydratation
ET	Ethylene
EST	Expressed sequence tag
FC	Fold change
GMS	Genic male sterility
GolS	Galactinol synthase
HI	Harvest index
HKGs	Housekeeping genes
InsP	Inositol phosphates
JA	Jasmonic acid
LEA	Late embryogenesis abundant proteins
m t	Million tonne
MAS	Marker-assisted selection
m ha	Million hectare
Mv	Mean value
NCED	9-*cis*-epoxycarotenoid dioxygenase
NGS	Next Generation Sequencing
OA	Osmotic adjustments
ORFs	Open reading frames
PCR	Polymerase chain reaction
PYLs	Pyrabactin resistance1-like proteins
PYR1	Pyrabactin resistance1
RCAR1	Regulatory component of ABA receptor1
RFLP	Restriction fragment length polymorphism
RFO	Raffinose family oligosaccharide
RIL	Recombinant inbreed lines
ROS	Reactive oxygen species
RSWC	Relative soil water content
QTL	Quantitative trait locus
tha^{-1}	Tonne per hectare
SA	Salicylic acid
SC	Self-compatibility, self-compatible
SD	Standard deviation
SE	Standard error
SI	Self-incompatibility, self-incompatible
SNF1	Sucrose non-fermentation 1
SWC	Soil water content
USPs	Universal stress proteins
UTR	Untraslated region
WUE	Water use efficiency
ZEP	Zeaxanthin epoxidase
ZFHD	zinc finger homeodomain

Acknowledgements

I would like to express my deep and sincere gratitude to my supervisor, Professor Vladislav Čurn, Ph.D., Head of the Biotechnological Centre, Faculty of Agriculture for his willingness towards my enrolment into a PhD programme at the University of South Bohemia in České Budějovice. He always given me an unlimited freedom that aroused my interest and driven me to acquire a much broader view of problems and approach them for different perspectives in other disciplines of plant sciences. His wide knowledge and his logical way of thinking have been of great value for me.

I am deeply grateful to Dr. Luigi Cattivelli, Ph.D., Director of CRA – Genomic Research Centre, Fiorenzuola d'Arda, Italy for his support and opportunity to gain valuable experience during my stay in CRA.

I wish to express my warm and sincere thanks to Dr. Caterina Marè, Ph.D., CRA – Genomic Research Centre, Fiorenzuola d'Arda, Italy for her co-supervision, encouraging, valuable suggestions, precision, understanding and personal guidance which provide me a good basis for the present thesis.

During this work I have collaborated with many colleagues for whom I have great regard and wish to extend my warmest thanks to all those who have helped me with my work in the Biotechnological Centre (České Budějovice) in the Czech Republic, CRA – Genomic Research Centre (Fiorenzuola d'Arda) in Italy, CRA – Cereal Research Center (Foggia) in Italy and CNR - IBBA, Research Institute of Agricultural Biology and Biotechnology (Milan) in Italy.

This thesis would not have been possible to finish without support from my friends Gio, Gabri, Elahe and Marketa.

The last *thank you* goes to my family, for allowing me to go and make my own mistakes.

The financial support from the University of South Bohemia in České Budějovice is gratefully acknowledged.

"Natural abilities are like natural plants; they need pruning by study. "

Sir Francis Bacon (1561 - 1626)

Chapter One

GENERAL INTRODUCTION

General Introduction

Human civilization is using breeding since almost outset beginning. Exploiting variation in the characteristics of the plant genetic resources through breeding is way, how to increase and diversify agricultural production and ensure its stability, adapt agriculture to ever changing environmental conditions and increasing social needs for food and other agricultural products and enhance in overall food safety. At the beginning, plant breeding or improvement was accomplished simply by selecting plants with one or a combination of the characteristic based on physical and agronomic attributes, alias their visible or measurable traits (phenotype) and reproducing preferred individuals or spontaneous variants, and indeed this practice remains important today as the basis for producing new generations of cultivated landraces and indigenous breeds. As the direct target of the selection was the trait itself and its phenotypic expression, genes forming genetic background or the specific genes behind the trait were selected indirectly. Some of these characteristics are influenced by the environment and are therefore not necessarily a good guide to the actual heritable genetic composition (genotype). Others may not be clearly visible or may be detected only in mature plants. Others again may be difficult or very costly to screen, and many characters such as drought tolerance are controlled by a large number of major and minor genes whose mode of action as well as their interaction with each other and with various environmental triggers is mainly unknown. Improving the identification, selection and monitoring of specific characters in plants through breeding schemes is therefore a critical need to secure future improvements in genetic resources for food and agriculture.

The main objective of present plant breeding is to improve the desirable crops in respect of different traits such us yield both simple and quantitative traits providing the foundations for modern genetics. Today's crops that are farmed are more or less the results of scientific breeding applied since the twentieth century, consisting of artificial

selection, application of Mendel's laws of inheritance and other breeding practices on wild life. Thanks to continuing investments made in research and technology development, the process of producing improved varieties, clones, breeds and strains of agriculturally important species has become progressively more accurate, now become possible to directly target genomic regions that are involved in the expression of traits of interest. Application of modern molecular techniques for study of plant genome becomes increasingly crucial for practical breeding and in comparison with classical selection based on phenotype with the above-mentioned disadvantages and limits, can provide genotype based selection and direct evaluation of its potential value. Structure and purpose of this thesis was to use modern molecular techniques to facilitate the standard procedures of breeding process.

Chapter Two

OBJECTIVES, STRUCTURE AND SUMMARY OF THE THESIS

Objectives of the Thesis

Considering the wide application of molecular techniques in research area, two major crops have been selected for the purposes of this dissertation for subsequent studies. The present thesis was composed of three main objectives from which each was subsequently divided into the three following sub-sections:

1. Identifying drought-induced genes in two durum wheat (*Triticum durum*) cultivars with contrasting response to drought stress.

1.1. Transcriptomic analysis based on an Affymetrix array platform of plants exposed to drought, heat and to combination of heat and drought.

1.2. Data analysis and identification of genes induced or more induced in one genotype compared with the other under the influence of drought stress.

1.3. Primer designing to detect candidate drought-induced genes and validate their expression profile in new experiment by qRT-PCR.

2. Expression QTL analysis of candidate drought-induced genes in durum wheat (*Triticum durum*) recombinant inbred lines (RILs).

2.1. Expression analysis of candidate genes carried out on RILs population to obtain quantitative data describing the expression profiles in response to stress.

2.2. Mapping of QTL controlling the expression of the drought induced genes.

2.3. Identifying of eQTL controlling the expression of stress induced genes, co-segregating between eQTLs and QTLs for drought tolerance evaluated on the bases of field trials.

3. Application of molecular markers for detection of self-incompatibility in oilseed rape (*Brassica napus*).

3.1. Screening of segregating F_2 population for SI plants by using available gene markers for *SLG*, *SCR*/*SP11* and *SRK* genes.

3.2. Validation of the molecular markers with phenotypic expression – seed production of the plants.

3.3. Comparative analysis of molecular markers with the aim to improve combination ability between them to refine selection of SI plants.

Summary in Czech – souhrn v češtině

V moderním šlechtění nalézají stále větší uplatnění techniky a přístupy využívající molekulární biologii. Koncepce této práce byla vymezena v podobě tří nezávislých studií, zaměřených na aplikaci dostupných molekulárních metod k analýze šlechtitelského materiálu.

První dvě studie jsou zaměřeny na studium změny genové exprese u dvou odrůd pšenice tvrdé (*Triticum durum*), Ofanto a Cappelli, s výrazně odlišnou odpovědí na stres suchem. Pro pochopení genetického pozadí reakce na stres suchem byla studována exprese ~50 000 genů pomocí Affymetrix microarray. Výrazně odlišné transkripční profily mezi dvěma odrůdami, společně s dostupnou anotací, vedly k detekci kandidátních genů, u kterých byl expresní profil ověřen v novém experimentu pomocí qRT-PCR analýzy. Vybrané kandidátní geny vykazující stabilní expresi byly použity ve druhé studii. V této studii byl expresní profil kandidátních genů testován na RIL populaci získané po křížení (rodičovských) odrůd Ofanto a Cappelli, reprezentující genetickou variabilitu celé segregující populace složené ze 161 linií. Cílem tohoto mapování bylo identifikovat expresní QTLs, objasňující mechanismus exprese vybraných kandidátních genů spojených s reakcí na stres suchem a jejich vazbu k rezistentním lokusům.

Třetí studie byla zaměřena na problematiku studia mechanismu účinku genů z tzv. „*S* lokusu“ a jejich vlivu na projev autoinkompatibility (AI) u řepky olejky (*Brassica napus*). K tomuto účelu byla použita segregující F_2 populace odvozená z donora recesivního typu autoinkompatibility a donora 00 kvality. S použitím specifických primerů pro geny *SLG*, *SRK* a *SCR*/*SP11* a funkční alely těchto genů byly provedeny molekulární analýzy, na jejichž základě byly vybrány potenciální AI rostliny. Přesnost a účinnost molekulárního screeningu byla hodnocena pomocí prověrky fenotypu všech analyzovaných rostlin tzv. „semenným testem“ (hodnocení počtu semen v šešulích po nuceném samosprášení). Tento metodický postup vedl k vývoji molekulárního selekčního markeru a k nalezení vhodné kombinace specifických primerů, které lze

využít v systému marker asistované selekce k detekci autoinkompatibilních rostlin v raných vývojových stadiích s vysokou přesností.

Tyto experimenty představují pouze malou část technik, které lze použít ke studiu rostlinného genomu, identifikaci genů řídících projev šlechtitelsky významných znaků a ke genotypově založené selekci. V rámci NGS technik poskytujících stále více informací o genomových sekvencích různých taxonů, lze do budoucna předpokládat stále se rozšiřující uplatnění přístupů využívajících molekulární biologie a jejich praktické aplikace v hodnocení a výběru šlechtitelského materiálu.

Summary in English

In modern breeding programmes, the integration of molecular biology still has increasing impact. The concept of this thesis was defined as three independent studies, focusing on the application of available molecular methods for analysis of breeding material.

The first two studies were focused on identification of genes differentially expressed in durum wheat (*Triticum durum*) cultivars Ofanto and Cappelli with significantly different responses to drought stress. To understand the genetic bases of drought stress response the expression levels of approximately 50 000 genes carried by the wheat Affymetrix microarray were investigated in two cultivars subjected to water stress. According to the significant differences of drought-induced gene transcripts and the annotations associated to these genes, drought response candidate gene markers were selected and their expression profile was validated by qRT-PCR.

Selected candidate genes with stable expression were used in the second study. To identify the expression QTLs in this study a RIL population (derived from the cross Ofanto and Cappelli cultivars) representing the genetic variability of whole segregating population of 161 lines was employed. The aim of this mapping was based on the integration of a genetic map, phenotypic and transcript level data to understand the relationship between resistance loci and the expression of stress responsive genes.

The third study was focused on understanding mechanism of action of genes belonging to the "S locus" and on investigating their impact on self-incompatibility of oilseed rape (*Brassica napus*). For this purpose the segregating F_2 population derived from donor of recessive type of self-incompatibility (SI) and donor of 00-quality was used. By using of specific primers for genes *SLG*, *SRK* and *SCR/SP11* and their functional alleles was carried out molecular analysis to detect putative SI plants. Accuracy and efficiency of molecular screening was evaluated by phenotypic expression (numbers of seeds per developed silique after self-pollination was counted). This methodology led to

developing selective molecular markers and to finding suitable combinations of specific primers that can be used in marker assisted selection system to detect self-incompatible plants in the early developmental stages with high accuracy.

These experiments represent only a small part of the techniques that can be used in plant genome studies, identification of genes important in plant breeding and genotype based selection. The application of NGS techniques allow considerably greater numbers of nucleotides to be characterized and so provides more information about genome sequences of different species. Using this novel approach is seen to increase continuously use of molecular biology across the future and their practical application in the evaluation and selection of breeding material.

Structure of the Thesis

The present thesis is designed with seven 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 transcriptomic and expression drought-related analysis, (Chapter 4), eQTL mapping (Chapter 5), marker assisted selection (MAS) (Chapter 6) and general review of drought stress on plants, where the durum wheat was used for experimental part of this study and other part of this review introduce impact of used molecular techniques for breeding of oilseed rape especially using self-incompatibility system for it (Chapter 3). In addition, general introduction (Chapter 1), summary (Chapter 2) and conclusion (Chapter 7) 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 explains the overall objectives, organization, arrangement of the topics and summary of the thesis.

Chapter 3 describes different plant strategy and response to the drought stress, which is followed by real drought-stress experiments on *Triticum durum* (genus *Triticum*) in chapter 4 and 5. Other part provides general information about molecular techniques which are currently used to accelerate the standard breeding procedures in *Brassica napus* (genus *Brassica*), especially for utilization of self-incompatible (SI) system followed by experiment in chapter 6.

Chapter 4 presents a work for the identification of drought-related genes differentially expressed in two cultivars with clear differences in response to heat and drought stress based on microarray analysis.

Chapter 5 describes expression QTL analysis of genes expressed in response to drought in a durum wheat RIL population. This chapter and also part of chapter 4 is part of a larger research work dedicated to the understanding of the molecular response of durum wheat to drought and heat stress. The overall work aims to identify genes induced in cultivars with contrasting stress tolerance levels and to explore the possibility of using expression data to find eQTLs in a segregation mapping population and link these findings to drought tolerance loci. It contains the manuscript in preparation.

Chapter 6 provides information about using specific molecular markers for self-incompatibility detection in oilseed rape (*Brassica napus* L.) and potential future perspective for future breeding. In this study we have used three *S* locus genes for selection of SI plants of segregating population derived from crosses between SI donor lines and self-compatible donors of 00-quality.

Chapter 7 explains the fulfilment objectives of the thesis, together with their experimental results and practical application for further exploitation.

Chapter Three

REVIEW OF LITERATURE

Review of Literature

DURUM WHEAT (*T. durum* Desf.) AND PLANT RESPONSE TO DROUGHT STRESS

Triticum durum and Plant Response to Drought Stress

1.1 Introduction

Cereals are an important commodity since the beginning of agriculture. Wheat is one of the main members of the tribe *Triticeae* which includes many domesticated species with some of the largest and complex genomes of the genomes of grasses (*Poaceae*[1]). *Triticeae* cereals (wheat, barley, and rye) represent major renewable resources for food, feed, and industrial raw materials and are the most important crops for European agriculture. The wheat group constitutes an alloploidy series containing diploid, tetraploid and hexaploid species with a basic chromosome set of x=7 (Belay *et al.*, 1994). The first evolutionary event which gave rise to polyploid wheats was hybridization caused by natural pollination of diploid wheat closely related to *Triticum urartu* (AA) a species closely related to einkorn wheat *T. monococcum* (AA) with a yet unknown species that provided the B genome and was closely related to *Aegilops speltoides* (Appendix 1; Biswas *et al.*, 2008). The subsequent genome duplication of hybrids by natural polyploidy gave rise to several wild and cultivated tetraploid species (2n = 28, AABB) like *Triticum dicoccum*, *T. timopheevii*, *T. turgidum*, *T. polonicum*. A second evolutionary event that led to the bread wheat lineage occurred when the early domesticated tetraploid durum wheat, *T. turgidum* ssp. *durum* (AABB) as a cytoplasm donor crossed with *A. tauschii*, a wild diploid species with a DD genome. 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 (Biswas *et al.*, 2008).

The most important wheat species grown currently are bread wheat (*Triticum aestivum*) and pasta wheat (*Triticum turgidum*) of which, hexaploid bread wheat, accounts for ~80% of world wheat production today (Table 1; FAOSTAT, data 2009)

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[1] The grass family *Poaceae* is the fourth largest family of flowering plants.

Durum wheat (*Triticum turgidum* Desf. var. *durum*, 2n = 4*x* = 28; AABB) is the only tetraploid species of wheat of commercial importance that is widely cultivated today. It has been selected from domesticated emmer wheat strains formerly grown in Central Europe and Near East, which developed a naked, free-threshing form. This species is the hardest of all wheats. Its high protein (at least 14%) and gluten content, as well as its strength, make the durum one, good for special uses, particularly pasta. The major durum wheat producing countries are UE, Canada, Syria, USA, Algeria and Morocco and minor production areas include Russia, Turkey, Tunisia, Mexico and India. In general is durum wheat cultivated on approximately 17 million hectares worldwide (De Vita *et al*., 2007).

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

Species	Production (m t)	Area (m ha)	Yield (t ha^{-1})	Percent total cereal production
Wheat	**681.9**	**225.4**	**3**	**27.4**
Barley	150.3	54.1	2.8	6
Rye	17.9	6.6	2.7	0.7
Triticale	15	4.1	3.6	0.6
Total Triticeae	865.1	290.3	3	34.8

*Data from Food and Agriculture Organization of the United Nations, FAO, 2009, (http://faostat.fao.org).

Given the predicted climatic changes and the fact, that even the most productive agricultural regions experience short period of drought within almost any year and occasional years with severe droughts, is important to ensure yield safety. The maximal biomass production and complete grain filling of major crop species including wheat in many parts of the world is often limited by abiotic stresses such as lack of available water necessary and extreme temperatures. High temperature is often attended by low water supply and therefore the primary aim of breeding must be to develop cultivars tolerant to both types of stresses (Tester and Bacic, 2005).

Drought tolerance is multigenic nature. During of breeding progress for high yield was indirectly improved yield in many water-limiting conditions (Araus *et al*., 2002, Morgan, 2000), but there is still a large gap between yields in optimal and stress conditions. Minimizing the “yield gap” and increasing yield stability under different stress conditions are of strategic importance in guaranteeing food for the future. Further progress will depend on the introduction in high yielding genotypes of traits able to

improve drought tolerance without detrimental effects on yield potential, thus reducing the gap between yield potential and yield in drought-prone environments. This goal can be achieved via the identification of drought tolerance-related traits and the subsequent manipulation of the corresponding genes using marker assisted selection (MAS) and/or gene transformation (Atienza *et al*., 2004). Obtained information from *Arabidopsis thaliana*[1], after determination of the genome sequence which was done in 2000 (Greilhuber *et al*., 2006) has allowed monitoring of expression profiles for all predicted genes at a single time by means of microarrays. This information has been used in functional analyses by using gene knockout mutants produced by T-DNA insertion via *Agrobacteriom* transformation or transposon movement (Kuromi *et al*., 2009) enhanced studies on abiotic stress responses and in other research fields. Also simultaneous progress via newly developing research fields such as discovery of functional small RNAs, such as micro RNA (miRNA) and small interference RNA (siRNA), has facilitated elucidation of the complex regulatory network in response to abiotic stresses (Sunkar *et al*., 2007). Now, the genome informations[2] from *Oryza sativa* and *Brachypodium distachyon* species close related to *Triticum* genus, facilitate comparative genomic studies to characterize the otholog genes in wheat.

1.2 Plant responses to abiotic stress

Abiotic stresses such as extremes in temperature, changes in the concentration of airborne gases[3], photon irradiance, and supplies of water and inorganic solutes or phytotoxic compounds, induce or repress the transcription of genes, which frequently limit growth and productivity (Bartels and Sunkar., 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). Those genes can be generally classified into two groups: those that are protecting against the stress by producing important metabolic proteins and cellular protectants and those that regulate signal transduction and gene expression associated with other processes (regulating genes) (Shinozaki and Yamaguchi-Shinozaki, 2000). The stress-inducible genes are involved in cell protection by producing important

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[1] *Arabidopsis thaliana* was chose as an ideal plant for genome sequencing for its reduced genome size and chromosome number (~157 Mb, *n* = 5) (Johnston *et al*, 2005), however this fact reduce its utility as a standard in comparative genomics (Schranz *et al*., 2006).

[2] www.phytozome.net and www.gramene.org

[3] Hypoxia or anoxia may cause cytoplasmic acidification, anaerobic fermentation or decrease in the membrane barrier function (Blokhina *et al*, 2003). Ozone significantly reduces photosynthesis in wheat (Biswas *et al*, 2008).

metabolic proteins and cellular protectants including osmoprotectants, membrane modifiers, turnover proteins or detoxification proteins (Way *et al*., 2005) and antioxidants (Hasegawa *et al*., 2000) while regulating genes (i.e. signalling molecules, transcription factors) are involved in control of expression of stress-inducible genes and therefore play a crucial role in the response to the stress (Barnabás *et al*., 2008). Genetic control of stress adaptation mechanisms is very complex system, consisting of the regulatory cascades of interactions within and between gene networks.

1.3 Successful strategies of plants to drought stress

There are three generally accepted strategy of plant resistant to drought: escape, avoidance and tolerance (for a review, see Chaves *et al*., 2003) and their combination. Escape strategies may rely on successful reproduction before the onset of severe stress. These plants show a high degree of developmental plasticity for their short life cycle, a higher rate of growth, gas exchange, the ability to use maximum of residual soil moisture (Chaves *et al*., 2003; Barnabás *et al*., 2008; Maroco *et al*., 2000; Amudha and Balasubramani, 2011) and ability to store reserves in organs such as stems or roots and use them for production of seed as it is happening in cereals (Bruce *et al*., 2000). Plants can withstand drought condition by avoiding tissue dehydration by maintaining tissue water potential as high as possible, or by tolerating low tissue water potential. Prevention of dehydration can be minimize water loss (i.e. stomatal closure[1], reduced leaf area by rolled or steep leaf angles, shedding of older leaves, higher concentration trichome layer) (Chaves *et al*., 2003, Larcher, 2000, Yordanov *et al*., 2003) or maximize water uptake (by root growth, increasing investment in the root, reallocation of nutrients stored in older leaves, and higher rates of photosynthesis) (Barnabás *et al*., 2008, Amudha and Balasubramani, 2011). Third strategy is based on tolerance to low water potential by assuring of plant function during lack of the water (osmotic adjustments (OA[2]), plant dormancy, rigid cell walls or cells reducing) or the recovery of plant water status and plant function after stress. Changes occurring rapidly at the mRNA and protein levels lead tolerant state (Ingram and Bartels, 1996). Some authors

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[1] The detailed mechanisms of stomatal response to drought are not easy to rationalise because at any given moment stomata may be responding to a complex set of factors ranging from light intensity to CO_2 concentration in addition to leaf water status.

[2] Osmotic adjustment (OA) is the net increase in intercellular solutes in response to water stress (Morgan, 1984).

mention the possibility of linking drought tolerance with efficient scavenging of reactive oxygen species (ROS[1]) formed as a consequence of disturbed metabolism (Sairam and Saxena, 2000). Recent research seems to imply that these species play a major role in plant defense, specially against pathogens (Mittler *et al*., 2004) while role of ROS in plant defense against insect herbivores like for drought tolerance is still not clear (Liu *et al*., 2010).

The whole-plant response to drought stress can be divided also from perspective of time as response caused by short-term responses or as long-term or acclimation responses (Figure 1). In the case of slowly developing water deficit (within days to weeks or months) have plants greater chance either escape dehydration by shortening their life cycle[2] or optimise their resource gain in the long term through acclimation responses. Short-term water deficit causes in plants minimising water lost or exhibiting metabolic protection against the damaging effects of dehydration and co-developing oxidative stress. Time of duration dessication can have totally different results in terms of physiological response or adaptation. Plants response may be also changed dramatically according to genotype and environment (Chaves *et al*., 2003).

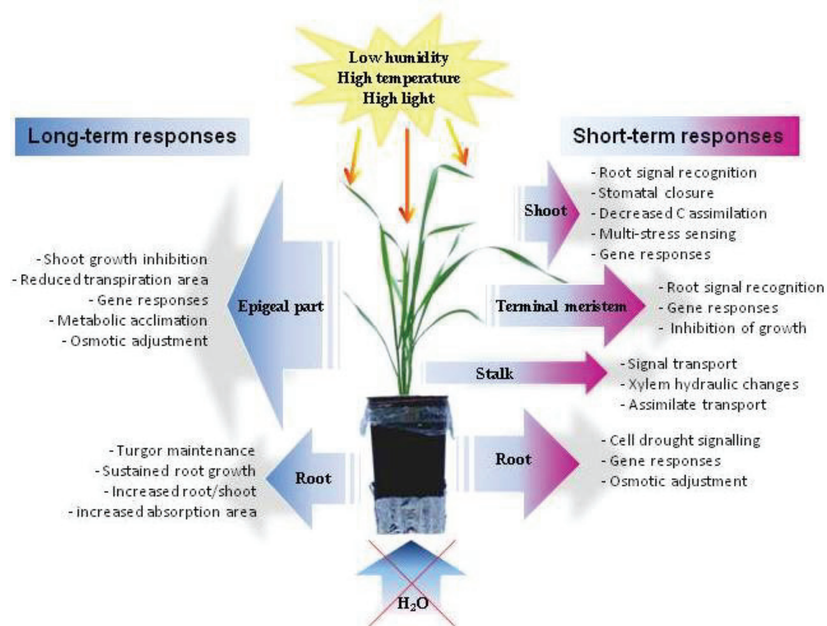

Figure 1 Whole-plant long-term (or acclimation) and short-term responses to drought stress (modified from Chaves *et al*., 2003); Pictured *Triticum durum* cv. Ofanto 49% Relative Soil Water Content (RSWC).

[1] The three major forms of ROS are: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical. These molecules are highly reactive and toxic and can lead to a hypersensitive response results in a zone of host cell death, which prevents further spread of biotrophic pathogens (for a review, see Liu *et al*., 2010).

[2] This strategy is important in arid regions for some native annuals.

1.4 Drought-inducible genes

In the plant soon after the perception by specific receptor(s) and recognition of external changes, different signaling pathways are activated in order to convert a physical stress into a biochemical response, each of them promoting the expression of a set of stress-responsive genes; the full activation of signal cascades induced by a given stress event promotes acclimation and leads to stress tolerance. Signal perception is followed by the generation of second messengers, like inositol phosphates (InsP) and Reactive Oxygen Species (ROS) which can modulate intracellular Ca^{2+} levels, often initiating phosphoprotein cascades that finally targets proteins directly involved in cellular protection or transcriptions factors which are controlling specific stress-regulated genes. The products of these stress-responsive genes may contain some regulatory molecules (abscisic acid (ABA), salicylic acid (SA), ethylene (ET) and jasmonic acid (JA)), which can (Ca^{2+} include), in turn, initiate a second round of signaling that may follow the above generic pathway, although different components are often involved (Xiong *et al*., 2002). Other existing molecules which are also critical, do not directly relay the signal, but are responsible in modification, delivery, or assembly of signaling components (Figure 2).

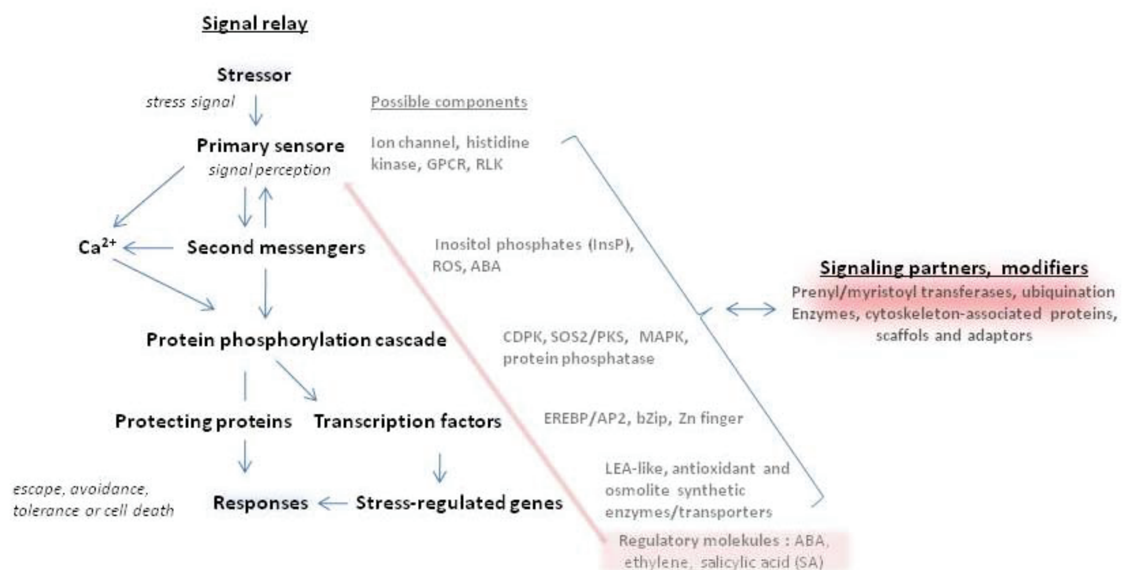

Figure 2 A general pathways for the transduction of stress signals caused by cold, drought, and salt stress in plants (modified from Xiong *et al*., 2002).

At present, hundreds of drought-inducible genes with various functions, including a number of transcription factors (Chen and Zhu, 2004) that regulate stress-inducible gene expression have been identified by molecular and genomic analyses.

Recently a number of these genes have been identified using microarray analysis in various plant species, such as *Arabidopsis* and rice. In relation to this a range of different techniques (gene expression patterns, transgenic plants, etc.) is being used to study their specific function and role in plant responses to water deficits (Hsieh *et al*., 2002; Ito *et al*., 2006). Plant responses to stress are complex[1] and the functions of many of these genes are still unknown (Shinozaki and Yamaguchi-Shinozaki, 2007). A transmembrane histidine kinase receptor (ATHK1)[2] and associated proteins forming a potential "osmosensor" have already been implicated in the perception of water deficit in *Arabidopsis* (Urao *et al*., 2001; Wohlbach *et al*., 2008). The stimulation of the "osmosensor", and/or other drought-sensing mechanisms, may initiate signal transduction cascades involving protein phosphorylation, dephosphorylation mediated by kinases or phosphatases up-regulated by water stress (Mishra *et al*., 2006). One early response to many different environmental stresses is a transient increase in cytosolic calcium concentration, released from the appoplastic space or internal stores (Knight, 2000), to mediate the integration of different signalling pathways (Kaur and Gupta, 2005) and as a second messenger to convert in most of cells external signal into cytosolic information (White and Broadley, 2003). To respond appropriately to a specific calcium perturbation, a cell must activate a unique combination of Ca^{2+}-binding proteins. These sensors include calmodulins (CaMs), CaM-like proteins, calcineurin B-like (CBL) proteins and Ca^{2+} dependent protein kinases (CDPKs). These proteins are termed cytosolic Ca^{2+} concentration "([Ca^{2+}]cyt) sensores". Several Ca^{2+}-dependent protein kinases have already been implicated in water stress-related signalling (Klimecka and Muszynska, 2007). Drought-activated kinase cascades finally result in the phosphorylation and activation of transcription factors regulating gene expression (Kaur and Gupta, 2005). Other second messengers like InsP and ROS can also modulate intracellular Ca^{2+} levels (Xiong *et al*., 2002).

[1] Adaptation to drought requires different gene expression for both, protection of cells against dehydration and whole-plant growth responses over longer time periods (Chaves *et al*., 2003). Signaling pathways constitute a complex network, interconnected at many levels (Knight and Knight, 2001). This explains the so-called "cross resistance" to different stresses and why plant responses to one particular stress can be elicited through several pathways (Bohnert and Shevelova, 1998).

[2] Equivalent receptor-type proteins which activate mitogen activated protein kinase (MAPK) have been identified also in yeast and in animals (Xiong *et al*., 2002).

The products of stress-inducible genes can be classified into two major groups: functional and regulatory proteins (Figure 3). However, Amudha and Balasubramani (2011) mention even third group so called "proteins with as yet unknown functions".

A. Functional proteins

This group includes for example water channel proteins functioning as "osmoprotectants", involved in movement of water through membranes. The enzymes synthesize various osmoprotectants or osmolytes such us sugars, proline or glycine-betaine, furthermore a number of sugar alcohols (mannitol, trehalose, galactinol, myo-inositol, sorbitol and raffinose[1]), quarternary and other amines (glycinebetaine and polyamines). Their presence provides a more efficient protection of the membrane and protein complexes during stress. Number of genes encoding enzymes that synthesize osmotic and other protectants are many, for instance Arginine decarboxylase (*adc*), Betaine aldehyde dehydrogenase (*BADH-1*), Choline oxidase (*codA*, *COX, CMO*), Osmotin protein accumulation (*Osm1*), Pyrroline carboxylate synthase (*P5CS*) etc. (for more details see Amudha and Balasubramani, 2011).

Another kind of proteins which may play a role in macromolecules and membrane protection are so called "LEA proteins". These include proteins with function molecules such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins and mRNA-binding proteins. LEA proteins represent another category of high molecular weight proteins that are abundant during late embryogenesis and accumulate during seed desiccation and in response to water stress (Galau *et al*., 1987). These proteins have 11-mer amino acid motifs with the consensus sequence TAQAAKEKAGE repeated as many as 13 times (Dure *et al*., 1993). LEA proteins are sorted into several groups and those proteins mentioned above belonging to group 3 and are predicted to play a role in sequestering ions that are concentrated during cellular dehydration. Into this group includes for example *HVA1* gene, where its overexpression confirmed improvement of growth characteristics and stress tolerance in wheat and rice experiment under salt- and water-stress condition (Sivamani *et al*., 2000; Rohila *et al*, 2002). For other groups is predicted to have enhanced water-binding capacity (group 1) or to sequester ions during water loss (group 5) (Amudha and Balasubramani, 2011).

[1] Synthesized by galactinol synthase (GolS) a key enzyme for osmolite biosynthesis involved in raffinose family oligosaccharide (RFOs) biosynthesis (include protecting cellular integrity during desiccation and/or imbibition, extending longevity in the dehydrated state (Downie *et al*., 2003)).

Greatest ability of group of “detoxifying genes” is scavenging of reactive oxygen species (ROS) providing higher stress tolerance and the accumulation of compatible solutes which may also protect plants against damage. Reactive oxygen species (ROS) are results of environmental stresses in most of the aerobic organisms and some are highly toxic and need to be rapidly detoxified. For the reason to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge the ROS. These antioxidant systems include various detoxification enzymes and non-enzymatic metabolites that may also play a significant role in ROS signaling in plants (Vranova *et al*., 2002). Into a group of enzymes involved in oxidative protection belongs for instance glutathione peroxidase, superoxide dismutase, ascorbate peroxidases or glutathione reductases (Amudha and Balasubramani, 2011).

Another strategy comprises improvement of photosynthesis under abiotic stress conditions through changes in the lipid biochemistry of the membranes and those genes which are involved in are so-called “multifunctional genes”. Higher content of unsaturated fatty acid in the membrane lipid composition may enhance adaptability of living cells for the cold temperature (Murakami *et al*., 2000).

A highly conserved biological response occurring in all organisms is heat shock response which includes induction of a set of genes in response to heat or toxic agent exposure so-called “heat shock protein genes”. The induction of genes encoding heat shock proteins (Hsps) is one of the most prominent responses observed at the molecular level of organisms exposed to high temperature (Vierling, 1991).

Recently were also diverse plant universal stress proteins (USPs) and new resource for their characterization identified, which can be use as useful targets for engineering plant cultivars tolerant to unfavourable environmental conditions (Isokpehi *et al*., 2011). Also is important to mention various proteases functions in abiotic stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007).

B. Regulatory proteins

To make plants more tolerant to stress a single gene encoding a single specific stress protein may not be every time sufficient to reach the required tolerance levels. For reason to enhance tolerance towards multiple stresses many studies of a gene encoding a stress inducible transcription factor that regulates a number of other genes and their protein products were recently processed (Krugman *et al*., 2010). The

regulatory proteins/factors are involved in further regulation of signal transduction and stress-responsive gene expression. These include various regulatory or signal transduction genes and transcription factors. Transcription factors have been identified to bind to promoter regulatory elements in genes that are regulated by abiotic stresses and activate cascades of genes that act together in enhancing tolerance towards multiple stresses. Individual members of the same family often respond differently to various stress stimuli, but otherwise also some stress responsive genes may share the same transcription factors resulting in overlapping of the gene expression profiles under different stresses (Chen and Murata, 2002). Further includes also protein kinases, protein phosphatases, alcohol or aldehyde dehydrogenase, enzymes involved in phospholipid, malate and NADP metabolism, signalling molecules such as calmodulin-, calcium-binding protein and so on (for details see Amudha and Balasubramani, 2011; Shinozaki and Yamaguchi-Shinozaki, 2007; Yamaguchi-Shinozaki and Shinozaki, 2005).

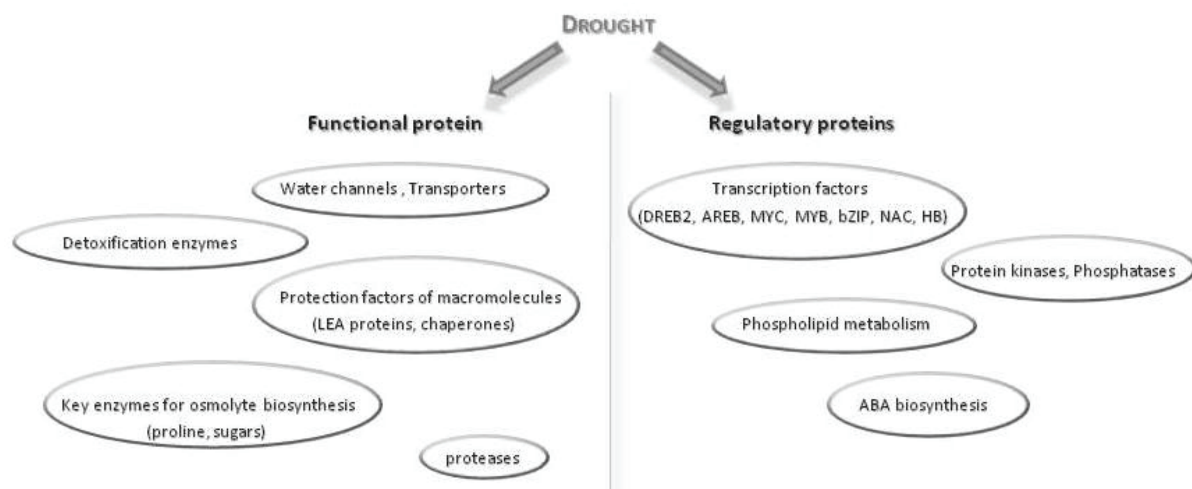

Figure 3 Drought stress-inducible gene products are classified into two major groups. One group encodes products that directly protect plant cells against stress (functional proteins), whereas the products of the other group regulate gene expression and signal transduction in stress responses (regulatory proteins), (modified from Shinozaki and Yamaguchi-Shinozaki, 2007).

1.5 Regulation of gene expression: ABA-independent and ABA-dependent regulatory system

There are currently two known regulatory systems governing drought-inducible gene expression, namely (phytohormone abscisis acid) ABA-independent and ABA-dependent regulatory system[1] (Yamaguchi-Shinozaki and Shinozaki, 2005; Hirayama and Shinozaki, 2010) (Figure 4). ABA is one of the long-term signals synthesized *de novo*

[1] This was confirmed by exogenous ABA treatment causing induction of several drought-inducible genes, whereas others were not affected (Boudsocq and Laurière, 2005).

primarily in response to drought and high salinity stress. Under non-stressful condition, ABA in plant cells is maintained at low levels which can be required for normal vegetative growth and cellular processes including seed development, dormancy and germination. Increased concentration of ABA in the root, induced by soil drying, may maintain root growth and increase root hydraulic conductivity. Both of these processes lead to an increase in water uptake and postpone the development of water deficit in the shoot. ABA is also transported in the xylem to the shoot, where it causes stomatal closure and reduces leaf expansion, to avoid dehydration of leaf tissues (Barnabás *et al*., 2008). ABA probably plays an important role in the mobilization reserves under drought condition (Yang *et al*., 2001), for example in the reduced plant water status and photosynthesis induces during grain filling the conversion of stem reserves into soluble sugars and the mobilization of sugars into the grains (Blum, 2005). The ABA signal transduction pathway probably includes a protein kinase/phosphatase cascade interacting with Ca^{2+} (Bray, 2002). The molecular mechanisms regulating gene expression in response to drought stress have been studied by analyzing both *cis*-acting and *trans*-acting regulatory elements functioning in ABA-independent and/or ABA-responsive pathways. Here we focus on recent progress in research on *cis*-acting elements involved in gene expression in response to drought, high salinity and cold stresses. Molecular response is composed of complex gene networks and stress-response cascades for specificity and cross-talk in abiotic stress-responsive gene expression based on interactions among *cis*-acting elements. The transcription factors interact with *cis*-acting elements in promoter regions and form a transcriptional initiation complex on the TATA box (core promoter) upstream of the transcriptional initiation sites. The transcriptional initiation complex then activates RNA polymerase to start transcription of stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 2005).

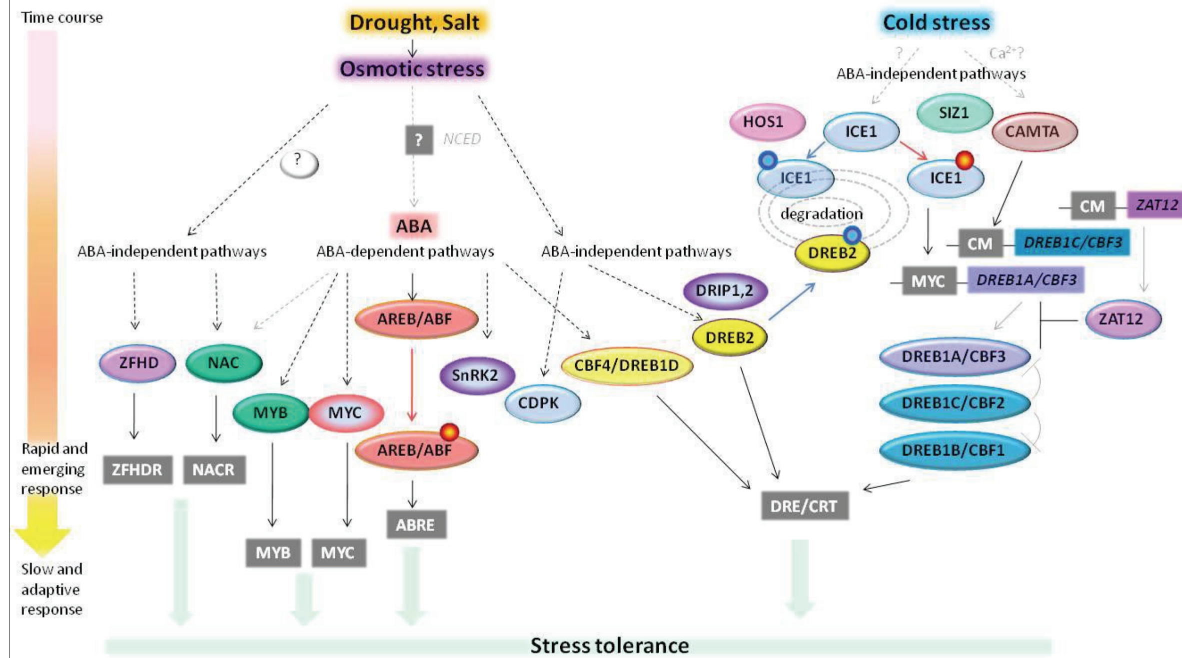

Figure 4 Transcriptional regulatory network of *cis*-acting elements and transcription factors involved in drought, salinity and cold stress responsive gene expression. Transcription factors controlling stress-inducible gene expression are depicted as ellipses. *Cis*-acting elements involved in stress responsive transcription are depicted as gray boxes. Small filled circles reveal modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Regulatory cascade of stress-responsive gene expression is shown from top to bottom. Early and emergency responses of gene expression are shown in the upper part and late and adaptive responses in the lower part. Solid black lines indicate the major direct signaling pathways. Dotted lines indicate indirect links. Colored lines indicate modifications. Gray dotted lines show uncertain connections. Abbreviations: ABA, abscisic acid; AREB, ABRE-binding proteins; ABRE, ABA-responsive element; CBF, C-repeat-binding factor; DRE/CRT, dehydratation-responsive element/C-repeat; DREB, DRE-binding protein; ICE, Inducer of CBF Expression; NACR, NAC recognition site; ZFHDR, zinc-finger homeodomain recognition site; CAMTA, calmodulin binding transcription activator, SIZ1, a SUMO E3 ligase (modified from Yamaguchi-Shinozaki and Shinozaki, 2005, Hirayama and Shinozaki, 2010). For details, see text.

ABA-dependent regulatory system

ABA – mediated responses

ABA is synthesised in the shoot and root in response to various stress. The first impulse to ABA biosynthesis can be assumed to start at the epoxidation of zeaxanthin, by zeaxanthin epoxidase (ZEP) to form epoxy xantophyll precursors (Taylor *et al*., 2000). While in leaves the steady-state levels of ZEP mRNA do not seem to be affected by dehydration, rapid increase (seven fold) in expression was observed in roots after 8-h dehydration period (Bray, 2002). Reason may be enough violaxanthin in leaves such that gene induction is not required for ABA biosynthesis, whereas in roots gene induction is essential to provide substrate for ABA biosynthesis (Bray, 2002). The key regulatory step in ABA biosynthesis in both roots and leaves is probably also through 9-*cis*-epoxycarotenoid dioxygenase (NCED). It was demonstrated that

overexpression of members of *NCED* gene family improves drought stress tolerance in transgenic *Arabidopsis* plants (Iuchi *et al*., 2001). NCED is an enzyme that converts the epoxycarotenoid precursor to xanthoxin in plastids (Qin and Zeevaart, 1999). Xanthoxin is then converted to ABA by two cytosolic enzymes via abscisic aldehyde. The *NCED* gene shown to have a promoter that is induced by drought both in leaves and roots, with evidence suggesting that regulation at the gene level is independent of ABA concentration (Chaves *et al*., 2003). Some experiments concerning overexpression of *PvNCED1* and *AtNCED3* genes respectively in tobacco (Qin and Zeevart, 2002; Iuchi *et* al., 2001) demonstrated that ABA levels can be manipulated, through the overexpression of this key regulatory gene in ABA biosynthesis, to improve drought tolerance. Otherwise, the overexpression of *LeNCED1* (from *Lycopersicon esculentum*) increased ABA levels in imbibed seeds and extended seed dormancy in tomato (Thompson *et al*., 2000). These experimental data suggest that ABA biosynthesis in developing and imbibing seeds may be regulated at multiple steps. The precise functions of regulation of ABA biosynthesis remain uncertain, but some studies suggested that ABA in higher plants is synthesized from an "indirect" pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA aldehyde and the final step, in ABA synthesis, is catalysed by the abscisic aldehyde oxidase (AAO) (Figure 5).

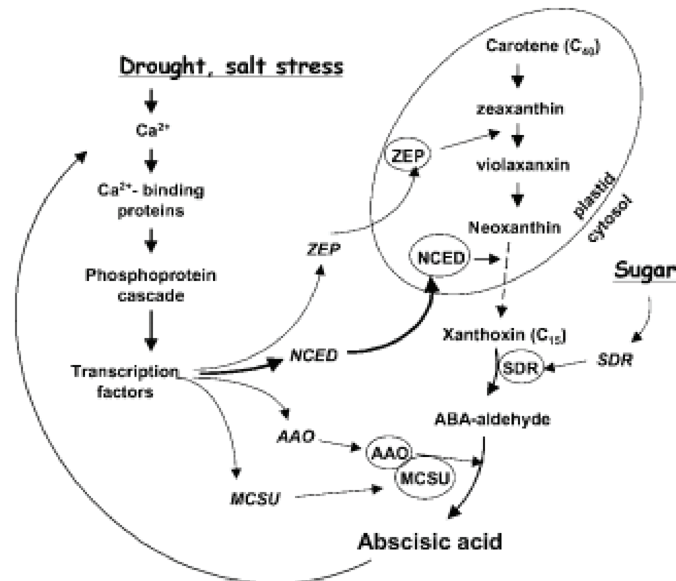

Figure 5 Regulation of ABA biosynthesis. ABA is derived from C_{40} epoxycarotenoid precursors through an oxidative cleavage reaction in plastids. The C_{15} intermediate xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde in cytosol. Abiotic stresses such as drought and salt activate the biosynthetic genes (italicized), probably through a Ca^{2+} -dependent phosphorelay cascade as shown on the left. ABA feedback stimulates the expression of the biosynthetic genes, which is also likely through a Ca^{2+}-dependent phosphoprotein cascade. Among the biosynthetic genes, NCED is strongly upregulated by stress (indicated with a thick arrow), whereas SDR is regulated by sugar. ABA biosynthetic enzymes are shown in small ovals. The NCED step probably limits ABA biosynthesis in leaves (indicated with a dashed arrow). ZEP, zeaxanthin epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; AAO, ABA-aldehyde oxidase; MCSU, MoCo sulfurase (Xiong and Zhu, 2003).

Recently, genes involved in ABA biosynthesis and catabolism were identified based on genetic and genomics analyses (Nambara and Marion-Poll, 2005). Promoter regions of several ABA-inducible genes were compared with each other and a conserved sequence, PyACGTGGC, was found in their promoter regions (Yamaguchi-Shinozaki and Shinozaki, 2005). This sequence was first identified as a *cis*-acting element named ABA responsive element (ABRE) in the wheat *Em* gene, which functions mainly in seeds during late embryogenesis (Guiltinan *et al.*, 1990), and in the rice *RAB16* gene, which is expressed in both dehydrated vegetative tissues and maturating seeds (Mundy *et al.*, 1999). This sequence is similar to a conserved *cis*-acting element called G-box (CACGTGGC), the region for several environmentally induced genes other than ABA (Menkes *et al.*, 1995). From recent studies two types of ABA receptors, soluble and membrane-anchored, have been reported. Pyrabactin Resistance1 (PYR1)/PYR1-like proteins (PYLs)/Regulatory Component of ABA Receptor1 (RCAR1)-type or START-type soluble ABA receptors were identified very recently using chemical genetic and biochemical approaches (for more information see: Ma *et al.*, 2009; Park *et al.*, 2009).

The *cis*-acting element in ABA-dependent gene expression is ABRE. ABA-reponsive transcription factors (AREB/ABF), with a bZIP type DNA-binding domain that binds the ABA-responsive element (ABRE) (T/CACGTGGC), have a pitoval role in ABA-dependent gene activation (Shinozaki and Yamaguchi-Shinozaki, 2007; Hirayama and Shinozaki, 2010). Several genes that are upregulated under drought conditions contain ABRE in their promoter regions (Uno *et al*., 2000). The AREB/ABF proteins require an ABA-mediated signal for their activation, as indicated by their reduced activity in the ABA-deficient *aba2* and ABA-insensitive *abi1* mutants and their enhanced activity in the ABA-hypersensitive *era1* mutant of *Arabidopsis* (Uno *et al*., 2000). This phenomenon is probably due to the ABA-dependent phosphorylation of the AREB/ABF proteins. The ABA-responsive transcription factors AB15 and AREB/ABF of *Arabidopsis* and TRAB1 of rice are regulated by the phosphorylation of multiple Ser/Thr residues (Hirayama and Shinozaki, 2010). Constitutive overexpression of ABF3 or ABF4/AREB2 in *Arabidopsis* caused ABA hypersensitivity, reduced transpiration rate, and enhanced drought tolerance (Kang *et al*., 2002). AREB1 was shown to be an essential component of induction of many ABA-responsive genes in transgenic plants without exogenous ABA application (Fujita *et al*., 2005). These data suggest that such constitutively active forms of transcription factors provided by point mutations may contribute to enhancement of drought tolerance in transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2007).

A MYC transcription factor, AtMYC2 and MYB transcription factor, AtMYB2, are synthesized *de novo* under osmotic stress conditions, and were shown to bind *cis*-elements in the RD22 promoter and co-operatively activate RD22 (Abe *et al*., 2003). The transcription factor AtMYC2 has been shown to be the convergent point of JA/ABA signal (Lorenzo and Solano, 2005). Also microarray analysis confirmed target genes of MYC/MYB in overexpressing transgenic plants, such as alcohol dehydrogenase and ABA- or JA-inducible genes (Abe *et al*., 2003). Overexpression of both AtMYC2 and AtMYB2 resulted in an ABA-hypersensitivity and improved osmotic stress tolerance of the transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2007).

A drought-inducible *RD26* gene encoding for a NAC transcription factor was identified in *Arabidopsis* (Fujita *et al*., 2004). Expression of RD26 NAC transcription factor gene is induced by drought, high salinity, ABA, and JA treatments. Mutant plant that overexpressed *RD26* gene was hypersensitive to ABA, and a *RD26* dominant repressor transgenic was insensitive to ABA. It was observed that ABA- and stress-

inducible genes were up-regulated in the RD26-overexpressing transgenics and repressed in the RD26 gene repressor lines. However, it was also discovered that many JA-inducible genes are target genes of RD26, while typical ABA-inducible genes such as LEA, RD, ERD, COR and KIN are not target genes of RD26 (Fujita *et al*., 2004). This indicates an important role for RD26 in mediating cross-talk between ABA signaling and JA signalling during drought and wounding stress responses (Shinozaki and Yamaguchi-Shinozaki, 2007).

Sucrose non-fermentation 1 (SNF1) related kinase family (SnRK1, SnRK2 and SnRK3 types) have been demonstrated to function in various stresses and ABA responses. Two SnRK1-type kinases (KIN10/SnRK1.1 and KIN11/SnRK1.1) are presumed to act in the regulation of metabolic pathways (Baena-González *et al*., 2007). Together with SRK2J/SnRK2.9, nine *Arabidopsis* SnRK2s are activated under osmotic stress conditions (Boudsoq *et al*., 2004). Among them, SRK2D/SnRK2.2, SRK2I/SnRK2.3 and SRK2E/OST1/SnRK2.6 are activated strongly by ABA. 10 members of SnRK2s in rice are significantly activated by drought, salinity, and ABA (Yoshida *et al*., 2002). Several type-2 SNF1-related protein kinases (SnRK2-type) were shown to mediate the regulation of stomatal aperture (e.g. SRK2E/OST1) and to function upstream of ABA-responsive expression (Mustilli *et al*., 2002, Yoshida *et al*., 2002). SRK2C is activated by osmotic stress, salt stress, and ABA treatment (Umezawa *et al*., 2004), is expressed in the root tip, and is involved in the root response to drought stress. In transgenic plants SRK2C improve stress tolerance, as many of the downstream genes it influences are stress inducible. In addition, SnRK2 protein kinases may activate transcription factors influencing osmotic stress-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007). In addition, a recent biochemical analysis succeeded in showing that clade A PP2Cs interact with ABA-activated SnRK2s and inactivate them efficiently through dephosphorylation at specific amino acid residues (Umezawa *et al*., 2009). Because SnRK2s can phosphorylate and activate some ABA-dependent transcription factors, some ABA signaling pathways have now been established from perception to gene expression (for details see Umezawa *et al*., 2009). Because the triple knockout mutant of three ABA-activated SnRK2 was extremly insensitive to ABA (Fujii and Zhu, 2009; Nakashima *et al*., 2009), this shows ABA receptor-PP2C-SnRK2 pathway as the major pathway (Hirayama and Shinozaki, 2010).

ABA-independent regulatory system

The major *cis*-acting regulatory element in ABA independent gene expression is DRE/CRT/LTRE. The RD29A/COR78/LT178 gene is induced by drought, cold and ABA. ABA-independent genes have a conserved DRE (dehydration responsive element with a TACCGACT consensus)/CRT (C-RepeaT). Deletion and base-substitution in the RD29A promoter have shown in transgenic plants experiment that the consensus sequence is an essential *cis*-acting element for regulating RD29A induction in the ABA-independent response to dehydration and cold in their promoters that is involved in gene regulation by interaction with an ABA-independent signalling cascade (Yamaguchi-Shinozaki and Shinozaki, 1994). Transcription factors belonging to the ERF/AP2 (APETALA2) family that bind to these DRE/CRT elements were isolated and described as CBF/DREB1 and DREB2 in previous work with conserved DNA-binding motif A/GCCGAC (Yamaguchi-Shinozaki and Shinozaki, 2005). According to contemporary model of regulatory network (Hirayama and Shinozaki, 2010), expression of DREB1A/CBF3 or DREB1C/CBF2 is regulated at the transcriptional level by Inducer of CBF Expression 1 (ICE1) (Chinnusamy *et al.*, 2003, Pellegrineschi *et al.*, 2004) or calmodulin binding transcription activator (CAMTA) (Doherty *et al.*, 2009), respectively. ICE1 is a MYC-type transcription factor that has also been shown to regulate stomata formation (Kanaoka *et al.*, 2008). This CAMTA transcription factors recognize Conserved Motif (CM) sequences providing a link with Ca^{2+} signaling that is activated in the abiotic stress response, as CAMTA proteins have a calmodulin-binding domain. ZAT12, a zinc finger protein, is also implicated in the regulation of DREB/CBF expression (Vogel *et al.*, 2005). DREB2, another AP2-type transcription factor that recognizes DRE/CRT, is involved in drought or salinity stress responses. Osmotic stress activates several other transcription factors, including zinc finger homeodomain (ZFHD) proteins and NAM, ATAF CUC2 (NAC). ZFHD1 binds the CACTAAATTGTCAC motif, named ZFHDR, in the promoter region of Early Response to Dehydration 1 (ERD1) (Hirayama and Shinozaki, 2010). DREB2 was shown to be regulated by DREB-Interacting Protein 1 and 2 (DRIP1 and DRIP2), which are RING finger E3 ligases, through ubiquitination (Qin *et al.*, 2008). ICE1 was demonstrated to be under the control of High Expression of Osmotically Responsive

gene 1 (HOS1), another RING finger protein (Dong *et al*., 2006). Not only ubiquitination[1], but also sumoylation is involved in the regulation of transcription factors in abiotic stress responses. In contrast to ubiquitination, sumoylation regulates the activity of target proteins. A recent study showed that a SUMO E3 ligase, SIZ1 sumoylated ICE1 and enhanced its activation of DREB1A/CBF3 (Miura *et al.*, 2007) Ca^{2+} -dependent protein kinase (CDPK) is good candidate for regulators of AREB/ABFss (Zhu *et al*., 2007). CDPKs are activated by increased intercellular Ca^{2+} levels induced by various stimuli (Harper *et al*., 2004) (for details see Figure 4).

1.6 Post-transcriptional gene regulation drought responses

The post-transcriptional regulations of gene expression of nuclear genes occur at the levels of pre-messenger RNA (mRNA) processing (capping, splicing and polyadenilation), mRNA stability, alternatively splicing, and mRNA translation efficiency (Floris *et al*., 2009; Krugman *et al*., 2010).

Regulation of mRNA processing

Precursor mRNA should be capped, polyadenylated and spliced before being translated into proteins. Alternative splicing allows production of more, than one mRNA from a single gene, for example ~30% of the *Arabidopsis thaliana* transcripts may be alternatively spliced (Matlin *et al*, 2005; Reddy *et al*., 2007). Several proteins acting as splicing regulators under stress conditions and particularly cold stress have been found (Palusa *et al*., 2007; Kim *et al*., 2007; Zhu *et al*., 2007a).

Regulation of mRNA stability by microRNAs and nat-siRNAs silencing

In the past few years was discovered, that some factors involved in abscisic acid signalling can be linked to the biogenesis of microRNAs (miRNAs), small RNA molecules that are important regulators of gene expression at the posttranscriptional level by repressing mRNA expression. In plants, miRNAs are 20-24 nt RNA molecules processed from non-coding dsRNA precursors by RNAs of the DICER-LIKE (DCL) family, that direct post-transcriptional regulation of gene expression through base-pairing to

[1] Protein ubiquitination and/or degradation by the ubiquitin/proteasome system (UPS) have been recognized as critical mechanisms in the regulation of numerous essential cellular functions (Si *et al*., 2008).

complementary regions in target transcripts. One strand of the mRNAs duplex is loaded into an Argonaute (AGO) protein to form a large multi-protein complex known as RISC (RNA-induced Silencing Complex), resulting in the inhibition of its expression by one of two general mechanisms: the mRNA can be destined to cleavage followed by further degradation or alternatively, miRNA recognition can lead to inhibition of mRNA translation (Covarrubias and Reyes, 2010). MicroRNAs are involved in numerous processes such as development, pattern formation, flowering time, hormone regulation, nutrient limitation, different forms of stress, and even self-regulation of the miRNA biogenesis pathway. (Jones-Rhoades *et al*., 2006). Recently was number of drought-related miRNA families in *A. thaliana*, *Phaseolus vulgaris* and *O. sativa* discovered (Liu *et al*., 2008; Reyes and Chua, 2007; Arenas-Huertero *et al*., 2009; Zhao *et al*., 2007, Dubozet *et al*., 2003). Is supposed, that accumulation of miRNAs would result in silencing of the target mRNAs and there is suggestion that it is required to remove them for an effective response to stress. There exist many conserved miRNAs in other plant species, but also less conserved miRNa which can participate in the regulation of processes particular to each species molecular and physiological mechanisms. The different plant strategies that have been selected in different plant species – and even between cultivars from the same species are making important to define the role of non-conserved miRNAs in stress responses. Blocking of translation does not cause cleavage of the target transcript upon miRNA binding, and therefore methods aiming at identifying the cleavage of mRNAs will overlook other potentially important miRNA-regulated mRNAs (Covarrubias and Reyes, 2010). For more details about miRNA sequences and their annotation miRBase is available on the website[1].

Natural antisense small interfering RNAs (nat-siRNA), has been identified from plants exposed to salt stress (Borsani *et al*., 2005). Some studies showed, that transcripts of some genes can be regulated under stress condition by nat-siRNA duplexes which can be incorporated into the RISC complex and cause inducing or silencing of these genes (Floris *et al*., 2009).

During the recent years, a new aspect of post-transcriptional regulation of gene expression via sequestration of mRNA in the cytoplasm has been studied (Weber *et al*., 2008; Anderson *et al*., 2009). The mechanism of mRNA sorting by localization of mRNA

[1] http://www.mirbase.org/

in distinct structures within the cytoplasm, and the resulting reversibile regulation of translation can be challenge for future studies on post-transcriptional regulation.

The informations mentioned above suggest that post-transcriptional regulation seems to be important for rapid adaptation of gene expression in the proteome as response to environmental changes, than transcriptional regulation can provide.

1.7 Translational regulation

Regulation of mRNA translation allows fine modulation of the level of protein synthetized from its corresponding mRNA. One mRNA can be read simultaneously by many ribosomes (forming so called polysomes) progressing along the mRNA to synthesize the same protein. The proportion of individual mRNA in polysomes can lead to a significant decrease in response to dehydratation stress (Kawaguchi *et al*., 2004). While some transcripts are up-regulated under stress, their association with polysomes is maintained at the same level as in normal conditions. The results suggest that the effect of dehydratation on translational level varied between mRNA species (Floris *et al*., 2009). In a microarray experiment of *A. thaliana* in response to sucrose starvation in cell culture were 224 of 25670 mRNAs transcripts translationally regulated as a consequence of general decrease of metabolic activity and related to cell cycle control (Nicolai *et al*., 2006). As was showed in experiment of Branco-Price *et al*. (2008) with *A. thaliana* seedlings under hypoxia stress, translational regulations can contribute to the adaptation of plants to environmental perturbation by limiting consumption of ATP and directing the synthesis of specific proteins. It is likely, that during environmental adaptation, many mRNAs undergo translational changes (Floris *et al*., 2009). It was also shown that translation of an mRNA is mostly affected by control elements located within untranslated regions (5’UTR and 3’UTR) and the interactions between them might promote a synergistic enhancement of mRNA translation, ensuring that scanning proceeds from the correct end. Some of the most important control signals in eukaryotes are upstream open reading frames (uORFs) that are located in the 5’UTR (Lovett *et al*., 1996). The presence of uORFs in 5’UTR modulates translation efficiency of the main ORF due to preferential recruitment of ribosomes through their reinitiation or leaky-scanning (Kozak *et al*., 2002; Wang *et al*., 2004). Some of uORFs provides translational regulation under stress condition including abiotic stress (Abastado *et al*., 1991; Floris *et al*., 2009).

Review of Literature

APPLICATION OF MOLECULAR MARKERS IN OILSEED RAPE (*B. napus* L.) BREEDING

Application of Molecular Markers in *Brassica napus* Breeding

2.1 Introduction

The family of the *Brassicaceae* (*Cruciferae*) includes many economically important crops species. It also includes the molecular plant model, such us *Arabidopsis* thaliana or rapid-cycling *Brassica* populations (Musgrave, 2000). The most important edible oil crop of *Brassicaceae* is canola or oilseed rape. *B. napus* (2n = 38, AACC) is of allotetraploid originated through spontaneous interspecific hybridisation between *B. rapa* (2n = 20, AA, chinese cabbage or turnip) and *B. oleracea* (2n = 18, CC, cabbage, brussel sprouts, cauliflower or broccoli) genomes. Both of these diploid species, together with third diploid species of U's triangle *B. nigra* (2n = 16, BB, black mustard) possess fully functionally self-incompatibility system, whereas *B. napus* and other two amphidiploids *B. juncea* (2n = 36, AABB, Indian mustard) and *B. carinata* (2n = 34, BBCC, Ethiopian mustard) generally occurs as a self-compatible plant (Downey and Rakow, 1987; Batley *et al*., 2007). The genomic relationships of the six *Brassica* crop species, known as U's triangle (1935) and shown in Figure 26 (Appendix 2), has been confirmed by chromosome pairing (for details see Figure 27), artificial synthesis of the amphidiploids[1], nuclear DNA content and sequence analysis, and the use of genome-specific markers (Warwick, 2011).

The only way how to preserve some important genetic traits for the future is maintain the current genetic pool and prevent further looses in next generations. In addition to this feature are natural breeding systems important also for affecting of homozygosity in organisms and have impact on levels and patterns of genetic diversity and genome evolution. Over the centuries, plants have evolved many adaptations that

1 The first successful hybrids have been produced by using sexual crosses (U, 1935, Morinaga, 1934) and fusion of protoplasts (Schenck and Röbbelen, 1982). The resythesis of *B. napus* provides an excellent model system (Sundberg and Glimelius, 1986) for comparisons between sexual an somatic hybridization because both systems can be used to produce hybrids from the same species parents.

encourage inbreeding (self-pollinaton/selfing) or outbreeding (cross-pollination/outcrossing) and many species of plants utilize a different variety of sexual and asexual breeding system. Due to the effect of plant breeding by humans, part of genetic variability also within oilseed rape breeding material was lost. The reason explaining the relative lack of genetic variability can be to a large extent also attributed to the limited geographical area, the Mediterranean region, where the natural habitats of the oil seed rape progenitor species overlap. The strategy, how to increase genetic basis of oilseed rape breeding material is using old landraces for future breeding providing some genetic potential which has been lost in current varieties or in the production of resynthesised rapeseed by crossing the original ancestors *B. oleracea* and *B. rapa* (Snowdon *et al*., 2007). Besides these techniques, interspecific hybridization can also be used to create novel genetic variation. A new approach to achieving this goal can be creating trigenomic hexaploid plants from tetraploid *Brassica* species (Tian *et al*., 2010; Pradhan *et al*., 2010).

2.2 Breeding methods and practices utilised in current breeding of oilseed rape

Before came the opportunity to use naturally occurring zero-erucic mutants (the 80's of 20th century) were in the Czech Republic mostly used landraces: Třebíčská krajová (released 1941), Slapská (1946) and Česká krajová (1949) belonging to the group “EG” with high content of erucic acid (~50%) and high level of glucosinolates (80-100μmol^{-1} in the seed). High quantity of erucic acid (*cis* 13-docosenoic acid, 22:1 n-9) in high doses can lead to cardiac damage and related health problems and also provides a bitter taste (Snowdon *et al*., 2007) General improvement in seed quality and high yield was followed also in several agronomic parameters such as cold-stress resistance, early maturity, suitability for machine harvesting, storing and mechanical resistance of the seed coats, resistance to weeds, insects and particularly, to the major diseases. Quality improvement of rapeseed oil as food stuff was succeeded by the development of “0” and “00” rapeseed varieties in the 1970s (Downey, 1990). Polish spring rape cultivar Bronowski was identified as a low glucosinolate form, and provided the basis for international backcrossing programmes to introduce this polygenic trait into high-yielding erucic-acid free material. The result was the release in 1974 of the

first 00-quality spring rapeseed variety Tower, with zero erucic acid and low glucosinolate content (Snowdon *et al.*, 2007). This was beginning for new 00-type oilseed rape variety in the following decades, also for the Czech Republic where new variety have been developed, such as: Sonáta (1990), Aglona (1993) Slapská Stela (1996) and Odila (1997) with up to 5% of erucic acid and content of glucosinolate up to 30µmol^{-1} in the seed. As “E0” oilseed rape containing high amount of erucic acid (~50%) with low content of glucosinolate (up to 25µmol^{-1}) for industrial purposes was variety Oáza released. Until now in the Czech national catalogue of varieties of agricultural species have been 11 spring and 91 winter oilseed rape varieties registered and for the other 9 spring and 144 winter oilseed rape varieties an application for entry have been submitted (ÚKZÚZ[1], data 2011). In ninetieth years of twentieth century was breeding focused on changes of fatty acid composition and presence of undesirable compounds like sinapic acid esters, phytic acid and phytates, phenolic acids and tannins. Some of the materials that are currently in the breeding process as future “000” types of rapeseed cultivars will be characterized by minimum content of erucic acid, reduced content of glucosinolate and also crude fiber (approx. from 12% to 6%) with yellow-seeded coats. Currently, the most promising route to reducing fiber and hull content genetically is to breed cultivars with a yellow (light) seed coat, like pure yellow-seeded cultivars occurring in the sarson subspecies of *B. rapa* or in *B. juncea*. Since yellow seed coats are significantly thinner than brown or black ones, the development of pure yellow-seeded *B. napus* cultivars with agronomically acceptable performance remains an important goal in quality breeding towards increased oil and protein content. These efforts especially in Chinese and Canadian breeding programmes led to the registration and release of new yellow-seeded hybrid cultivar “Yuhuang No 4” and line “YN01-429” respectively. Another aim of the breeders is also decrease content of α-linolenic acid (18:3n-3) which is more liable to rapid oxidative damage than oils with little or without 18:3n-3 content (Snowdon *et al.*, 2007).

In 2009 world production of oilcrops have been 808 m t, with soybeans dominanting (222.3 m t), followed oil palm fruit, seed cotton and rapeseeds (Table 2; FAOSTAT data 2009). Main annual rapeseed production and therefore a permanent

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[1] Data from Central Institute for Supervising and Testing in agriculture, ÚKZÚZ, obtained on-line 12/04/2011, (http://nou.ukzuz.cz/ido/).

position on the top have had during the 2000-2007 period China[1] followed by Canada[2] (~11.6 and ~7.4 m t respectively). In 2008 Canada reached first place in worldwide rapeseed production, followed by China. During this period (i.e. 2000-2008), the Czech Republic occurred between 8-10 position in worldwide rapeseed production with average of 0.84 m t production (FAOSTAT[3], data 2000-2008). The world average yield of 1.98 m t per hectare in 2009 covered a wide range, with up to 4.61 t/ha in Netherlands, 1.87 in China, 1.94 in Canada and 1.37 in Australia to 0.62 t/ha in Tunisia or 0.38 in Tajikistan (FAOSTAT, data 2009). These differences are due to varieties (e.g. winter vs. spring type), climate and soil as well as the use of agricultural inputs (seed quality, fertilizers and agrochemicals) and different agronomic technologies.

The majority of oilseed rape cultivars are pure lines derived from breeding schemes designed for self-fertilizing crops (i.e. pedigree selection or modifications thereof). Backcrossing has been successfully used to transfer simply inherited trades such as low erucic acid and glucosinolate content into adapted breeding material (Snowdon *et al*., 2007). In breeding techniques of *B. napus* was also used knowledge from biotechnology for good amenability of this crop with this kind of techniques. For example the utilisation of doubled haploid techniques in rapeseed breeding by using of anther and later by microspore culture for doubled haploids production (Kučera *et al*., 2002). Those haploid techniques provide rapid fixation of segregating genotypes, occurring in lower frequency, in which recessive genes coding for specific traits are combined in the homozygous condition. Thus, utilisation of microspore culture can allow a substantial acceleration of the breeding cycle for the improvement of the effectiveness of breeding new productive cultivars. Due to the generally high response of *B. napus* genotypes, the use of DH production has become common practice in commercial breeding programs and has already resulted in numerous registered cultivars.

Besides haploid techniques, wide hybridizations followed by embryo rescue techniques or protoplast fusion can also be used to create novel genetic variation. However, once a useful property has been identified in a basic breeding stock, e.g. a

[1]For this comparison is not include European Union (EU-27) as one unit - (in this case should be EU-27 on first place of worldwide rapeseed production).
[2]There is one exception for Canada in year 2002 - in this year had India higher level of production. India otherwise occupy always third place in worldwide production of rapeseed during the 2000-2008 period.
[3] Data from Food and Agriculture Organization of the United Nations, FAO, obtained on-line 12/04/2011, (http://faostat.fao.org).

mutant line or germplasm from a wild relative, it may take many years to accomplish the development of cultivars possessing this novel desirable trait. Marker-assisted selection (MAS) has had a significant impact on the efficiency of plant breeding routines such as backcrossing programs. In cases where conventional approaches have not been sufficient, further improvements can be achieved by genetic engineering. (Snowdon *et al*., 2007).

Table 2 Global production and yield of major oilseed crops*.

Species	Production (m t)	Area (m ha)	Yield (t ha^{-1})	Percent total oilcrops production
Soybeans	222.3	98.8	2.2	27.5
Oil palm fruit	207.3	14.7	14.1	25.7
Seed cotton	64	31.2	2.1	7.9
Rapeseed	**61.6**	**31**	**2**	**7.6**
Coconuts	59.9	11.2	5.3	7.4
Total Oilcrops	808.1	260.2	3.1	100

*Data from Food and Agriculture Organization of the United Nations, FAO, 2009, (http://faostat.fao.org).

2.3 Hybrid breeding, self-incompatibility and cytoplasmic male sterility system

The proportion of production of hybrid cultivars in comparison to the conventional open-pollinating varieties in the Czech Republic has led to a major increase withing the last few years. According to information of SPZO[1], was in 2010 harvested 48.6% of hybrids. Current hybrid systems declare yield improvements of up to 15% in F_1 hybrids compared to non-hybrid open-pollinating varieties. In fact this number is probably lower (i.e. less than 10%) due to the selected intensity of cultivation. Hybrids in addition show better vitality during germination, better drought resistance, faster recovery in the spring, require lower sowing rate per unit area and higher amount of branches and seeds. Otherwise acquisition costs of commercial hybrid cultivars are higher, small plants are more sensitive to the cold, plant have uneven ripening, produce large quantities of biomass and may contain higher amounts of glucosinolates. Hybrid breeding and subsequent seed production requires following steps: homozygous lines productions (by self-pollination, artificial doubled haploid (DH) production), testing

[1] The information obtained on-line 06/06/2011 (http://www.spzo.cz/).

combining ability of these lines and prevent self-pollination of the female parent line. To protect plants from self-pollination castration (used for maize, poppy and some vegetables, but not for oilseed rape), self-incompatibility (SI) or cytoplasmic male sterility (CMS) is used.

Self-incompatibility in Brassica species is controlled by a single (multiallelic) dominant genetic locus called the *S* locus (Bateman, 1955) and is of the sporophytic type. The self-incompatibility response relies on a series of complex cellular interactions between the self-incompatible pollen and pistil and represents the most widespread strategy preventing inbreeding and promoting outcrossing. In sporophytic self-incompatibility system is self-incompatible behaviour of pollen determined by the genotype of the pollen parent. SRK, SP11/SCR and SLG are the determinants that initiate the allele-specific rejection of self-incompatible pollen. SLG and SRK are female determinants encoding S proteins in the pistil (Silva and Goring, 2001). The extracellular domain of SRK shares a high degree of sequence similarity with SLG implying that both genes co-evolved and that SLG perhaps is the result of a duplication of the SRK extracellular domain. SRK is primary determnant of self-incompatibility in the pistil, however, the claim for SLG in this response has been somewhat controversial. Several tightly linked genes that play a role in controlling self-incompatibility have been identified in the *S* locus region. The term S haplotype has been adopted to designate the different *S* alleles for this group of multi-allelic genes in the *S* locus region. Since both SLG and SRK are highly polymorphic, the S haplotypes of both genes can be divided into two classes based on sequence similarities between the SLG proteins and between the SRK proteins. Class I *S* haplotypes possess highly polymorphic *SLG* and *SRK* genes, and show a strong self-incompatibility phenotype. Class II *S* haplotypes, however, possess *SLG* and *SRK* genes that are less polymorphic, exhibit a weaker self-incompatibility phenotype and are recessive to class I alleles. The male determinant of self-incompatibility, termed SP11 or SCR, was identified at the same time by Suzuki *et al.* (1999) and Schopfer *et al.* (1999) and is expressed in anthers. However, other components are required to carry out cellular responses leading to pollen rejection such us THL1, THL2 or ARC1 proteins (Silva and Goring, 2001). Interesting is also occurence of some dominant and recesive interactions between *S* alleles, because many of the *S* alleles are codominant. How does the dominant–recessive relationship between these *S* haplotypes emerge was described by Tarutani *et al.* (2010) in recent study of small

non-coding RNA (sRNA) controlling methylation of promoter regions of recessive *SP11* alleles in the anther tapetum.

Numerous cytoplasmic male sterility (CMS) systems are used in *Brassica* crops (*nap*, *ogu*, *pol, Shaan 2* and *tour*). CMS has arisen from specific interactions between the mitochondrial and nuclear genomes and the combination of cytoplasm and nucleus from different species (via interspecific crosses) often results in complete or partial male or female sterility and in many cases functional mutations of floral structure, leaf chlorosis and other defects that can appear together with CMS (Bannerot *et al*., 1977; Edwardson, 1970). As with many other forms of CMS, the mtDNA regions implicated in specifying the *nap* and *pol* forms of spontaneous male sterility found in *B. napus* contain novel open reading frames (ORFs) that can be physically associated and co-transcribed with standard mitochondrial genes (Schnable and Wise, 1998). Restorer genes are present in response to selective pressure created by the spread of male sterile cytoplasm in a plant population and can be also used to differentiate CMS system within the same species indicating that restoration of fertility is specific for each CMS system (Bellaoui *et al*., 1999) The nuclear genes that restore fertility to male sterile *nap* and *pol* cytoplasms (*Rfn* and *Rfp*, respectively) were found to represent different alleles or haplotypes of a single nuclear locus. Both alleles specify factors that influence mtRNA processing events, but the specific processing events conditioned by the two alleles are different, suggesting that the factors encoded by these genes recognize distinct RNA structural features (Li *et al*., 1998). Unlike other nuclear genes that affect mitochondrial gene expression, *Rfn* is capable of modifying the expression of multiple mtDNA regions, some of which are not associated with CMS (Brown, 1999). Most *B. napus* varieties contain the *nap* cytoplasm but are male-fertile because they possess a restorer gene for *nap* CMS, designated here as *Rfn* (Fan *et al*., 1986). The *nap* cytoplasm confers male sterility on a few exceptional varieties that lack *Rfn*, such as the cultivar Bronowski (Thompson, 1972). The male fertile maintainer strains of these varietes contain the fertile *cam* cytoplasm derived from the related species *Brassica campestris* (L'Homme and Brown, 1993). Most *B. napus* varieties lack a restorer gene for *pol* CMS and, hence, are sterilized by *pol* cytoplasm (Fan *et al*., 1986). Restorer genes for *pol* CMS have been identified in various strains (Fan *et al*., 1986) and map to a single nuclear locus designated *Rfp* (Jean *et al*., 1997).

Other *B. napus* CMS system is the widely used Ogura-INRA originating from interspecific crosses *Raphanus sativus* (Ogura, 1968), which was transferred to oilseed

rape by French scientists some 37 years ago (Bannerot *et al*., 1974). The *ogura* cytoplasm also confers male sterility to *B. juncea* (Labana and Banga, 1989) and CMS *juncea* promotes CMS in *B. napus* (Liu *et al*., 1996). In the case of *B. oleracea*, CMS plants were obtained by transfer of the CMS *B. juncea* cytoplasm from *B. rapa* through protoplast fusion (Cardi and Earle, 1997). Although this system was described by Tokumasu (1951) as a genic male sterility (GMS), in *B. napus* it is expressed as CMS. The Ogura CMS in radish (*Raphanus sativus*) is caused by an aberrant mitochondrial gene, *orf*138, that prevents the production of functional pollen without affecting female fertility. *Rfo*, a nuclear gene from radish that restores male fertility, alters the expression of *orf*138 at the posttranscriptional level. Analysis carried out by Desloire *et al*. (2003) confirmed that the *Rfo* gene is a member of the pentatricopeptide repeat (PPR) family. In *Arabidopsis*, the PPR gene family contains more than 450 members of unknown function, most of which are predicted to be targeted to mitochondria and chloroplasts and are thought to have roles in organellar gene expression (Desloire *et al*., 2003; Snowdon *et al*., 2007).

Using of CMS systems in oilseed rape breeding, however, has encountered certain difficulties in the development of suitable restorer or maintainer lines free of the negative agronomic characters and can be often inhibited by instability, or negative effects of the cytoplasm used to induce the male sterility (Snowdon *et al*., 2007). Environmental instability of the expression of *nap* male sterility shows that the use of this system is unsuitable for hybrid production. On the contrary, monogenically inherited restorer genes for *B. napus* "Polima" CMS identified by Dr. Fu in the Polima cultivar can be readily introduced into elite lines and *pol* is therefore now effectively used to produce registered F_1 hybrid spring canola varieties in numerous countries. Restored F_1 hybrids based on the Ogura CMS system are under increasing production in France and other European countries (~40% of the hybrids), and hybrid cultivars based on the commercial Male-Sterility Lembke (MSL) system are currently among the best-selling winter oilseed rape varieties in Germany. In Canada and Europe, approximately 60% of hybrid seed is prodused using the nuclear SeedLink™ system (Havey, 2004). This targeting tapetum technology was developed in the 1990s by a collaborative effort between the Goldberg group at the University of California and Belgium company Plant Genetic System (PGS), using the barnase-barstar system. SeedLink™ system is currently the only transgenic system used for comercial hybrid seed production (Kempe and Gils,

2011). Nuclear male sterility of the first parental line (MS1) results from the localized production of the bacterial RNAse gene *barnase* (obtained from *Bacillus amyloliquifaciens*; Paddon and Hartley, 1986) in a specific anther cell layer, and at a specific stage in anther development. Fertility restoration in the hybrid line is obtained by insertion, in the second parental line (RF1) of a gene coding for tapetal cell-specific ribonuclease inhibitor *barstar*, a specific inhibitor of the enzyme *barnase*.

2.4 Genetic diversity in *Brassica napus* and perspectives for its increase

Current relative lack of genetic variability in modern comercial cultivars and breeding materials of oil seed reape is due to the limited geographical area (i.e. for all Mediteranean region are progenitor species overlapping), lack of known local landraces (i.e. for winter oilseed rape are only 3 distinct) and incorporation of 00 trait into rapeseed varieties from single sources (i.e. the spring cvs. Liho and Bronowski). The efforts how to increase the narrow gene pool of present-day is the production of resynthesised rapeseed by crossing the original ancestors *B. oleracea* and *B. rapa*. This strategy can not only increase genetic variability with a view to hybrid breeding but also to broaden the genetic base with respect to pest and disease resistances, which in some cases is severely eroded in *B. napus*. Resynthesised rapeseed also represents an interesting source of genetic variation for quality improvement in oilseed rape or seed-color. To break down incompatibility barriers during interspecific hybridizations a variety of biotechnological tools as embryo rescue techniques or protoplast fusion can be used. During sexual hybridization it was also observed that also possible combinations between *B. juncea B. napus* and *B. carinata* including reciprocal crosses can has impact on plants vigor (Rao, 1990). Finally, induced mutagenesis have been used for creating new variability hitherto not available by utilizing of ionizing radiation (X-rays and cobalt 60) and chemicals such as ethylemethane sulfonate (EMS) (Rai *et al*., 2007).

2.5 Marker assisted selection of self-incompatibility

Marker Assisted Selection (MAS) is a combined product of traditional approaches and molecular biology which does not replace traditional breeding, but can help to make it more efficient. This method offers tools for targeted selection of the existing plant material for further breeding. MAS has already proven to be a valuable tool for plant breeders: it requires less investment, raises fewer safety concerns, respects species barriers, and is accepted by the public because does not include the transfer of isolated gene sequences such as genetic engineering. However, despite the considerable resources that have been invested in this field and despite the enormous potential it still represent, with few exceptions, MAS has not yet delivered its expected benefits in commercial breeding programmes (Ruane and Sonnino, 2007). This technique allows selection of genes that control traits of interest. Combined with traditional selection techniques, MAS has become a valuable tool in selecting organisms for traits of interest, such as color, content quality, or disease resistance, in identifying specific genotypes (i.e. genotyping), quantifying genetic diversity and relationships within and between populations or investigating biological processes. Molecular markers (identified as genetic markers) are used in molecular biology and biotechnology experiments for the classification of germplasm and in MAS (Edwards and McCouch, 2007). The relationship between the markers and genes of interest can be as described below. Some molecular markers have no know function or impact on plant performance (marker is not in linkage disequilibrium (LE)), other markers so called *direct markers* (Dekkers, 2004), may be used to find genes of interest[1], because they involve the gene of interest itselfs. In this case, one can refer to gene-assisted selection (GAS). Between these two types of markers exists also molecular markers called *linked markers* located very close to major genes of interest, but they are not part of it (marker is in linkage disequilibrium (LD)). This kind of molecular marker is said to be linked to that gene (Ruane and Sonnino, 2007). However, if scientists studied the progeny (offspring) of the parents through many generations, they may determine the presence of a useful molecular marker. Different kinds of molecular markers exist, such as polymerase chain reaction (PCR)

[1] The gene of interest is directly related with production of protein(s) that produce certain phenotypes whereas markers should not influence the trait of interest but are genetically linked (and so go together during segregation of gametes due to the concomitant reduction in homologous recombination between the marker and gene of interest). However, if a gene is not isolated marker's help is taken to tag a gene of interest. In such case there may be some false positive results due to recombination between marker of interest and gene (or QTL). A perfect marker would elicit no false positive results.

(Mullis *et al*., 1986) based markers as random amplified polymorphic DNA (RAPDs) markers (Williams *et al*., 1990; Welsh and McClelland, 1990), or its combination with digestion of amplified DNA so called polymerase chain reaction fragment length polymorphisms (PCR-RFLPs) (Botstein *et al*., 1980) based on restriction fragment length polymorphisms (RFLPs). Molecular markers derived from the selective amplification of restriction fragments are called amplified fragment length polymorphisms (AFLPs) (Vos *et al*., 1995) and can be modified by using a microarray platform as diversity array technology (DArT) (Jaccoud *et al*., 2001), Further molecular markers based on DNA sequence information have been developed. Into this group can be placed molecular markers such as, microsatellites (MS, STRs, SSRs), inter-SSR markers (ISSRs), transposable element-based markers (TEs), rDNA (ribosomal DNA sequences), single nucleotide polymorphism (SNPs), single feature polymorphisms (SFPs) and Affymetrix-type arrays (Edwards and McCouch, 2007).

Chapter Four

USING OF MICROARRAY TECHNOLOGY FOR IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN TWO DURUM WHEAT CULTIVARS WITH CONTRASTING RESPONSE TO DROUGHT STRESS BY QRT-PCR

Identification of Drought-induced Genes in *Triticum durum* by qRT-PCR

3.1 Abstract

Quantitative real-time polymerase chain reaction (qRT-PCR) and microarray analysis have become essential for clarifying variations in gene expression. The durum wheat cultivars "Cappelli" and "Ofanto" are characterized by contrasting behaviour in response to high temperature and drought stress, as well as having different water use efficiency (WUE). A microarray experiment was carried out in the two cultivars subjected to water stress at booting stage to identify the genes involved in the molecular response to drought stress. Differentially expressed genes showing different stress expression profiles were found in Cappelli vs Ofanto flag leaves samples (Aprile *et al*., in preparation). According to the fold change threshold of the Affymetrix dataset and the annotated activity among these genes some drought response gene markers were selected. Some gene markers have been confirmed by qRT-PCR to maintain a different expression profile between Ofanto and Cappelli during a new experiment performed at tillering developmental stage in drought-related conditions. These results will open the possibility to identify expression QTLs[1] controlling the response of durum wheat to abiotic stress making possible the understanding of the genetic bases of stress dependent gene expression.

Key words: *Triticum durum*, drought stress, expression analysis, RSWC, Microarrays

[1] Quantitative Trait Locus (QTL) mapping is a set of techniques that locates genomic loci associated with phenotypic variation in a genetically segregating population.

3.2 Introduction

Wheat is an important crop and durum wheat is widely grown in Mediterranean climates for the production of pasta, bread and other products. Drought is the major abiotic limitation to crop yield with significant agricultural, economic, political and social impact, especially since durum wheat is mainly farmed under rainfed environments. Improving drought tolerance without detrimental effects on yield potential appears as an urgent imperative in a scenario of increased need to save water.

A number of stress-inducible genes have been identified in wheat using microarray analysis (Aprile *et al*., 2009; Krugman *et al*., 2010). A new transcriptomic experiment based on microarray analysis was carried on flag leaves at booting stage with the Affymetrix 61K wheat chip on two durum wheat cultivars with contrasting response to drought and heat stress to understand the genetic bases of drought stress response. Ofanto is a modern cultivar characterized by high harvest index (HI), high yield and yield stability in moderate drought stress condition. Cappelli is an old cultivar characterized by low harvest index (HI) and low yield. These cultivars were selected for their contrasting behavior in response to high temperature and drought stress. The expression levels of some genes carried by the wheat Affymetrix microarray were investigated in the two cultivars subjected to water stress at booting stage (Aprile *et al*., in preparation).

In this work we proposed to identify some drought and heat response genes differentially expressed between the two cultivars and validate their expression profile in different experiments and developmental stage. Based on the most promising results, some genes will be used in further studies to test a RILs population and their expression data as quantitative trait to map stress-responsive expression QTL taking advantage from an existing molecular marker map.

3.3 Materials and methods

3.3.1 Microarray approach for QTL gene discovery

To understand the genetic bases of drought stress response, a transcriptome analysis was carried out using the Affymetrix 61K microarray wheat chip on mRNA extracted from flag leaves from the two cultivars subjected to water stress at booting stage. Plants, grown in optimal controlled conditions till booting stage, were exposed to

three different stress treatments: drought, high temperatures and a combination of both stresses. For drought stress, the water stress was imposed by withholding water and by bringing the pots to 12.5% of Soil Water Content (SWC), maintaining the conditions of temperature, relative humidity and photoperiod typical of the booting stage. To monitor the SWC, the weight of the pots is measured twice per day. Samples, consisting of the flag leaf detached at mid-point of light of sampling day, were taken one, two and three days after the imposition of stress. For heat stress, the plants were exposed to a gradual increase of temperature with the following temperature profile: 25 °C day/18 °C night for 24h - 30 °C day/22 °C night for 24h - 34 °C day/26 °C night for 48h.

In microarray experiment, RNAs from well-watered flag leaves collected at booting stage (SWC>95%) were used as reference samples and compared against the RNAs from flag leaves from plants subjected to stress initiated at booting (SWC=12.5%). All experiments were carried out in triplicate. Leaf material used for molecular analysis was immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction.

To find statistically relevant differentially expressed genes a Welch t-test with Benjamini and Hocberg correction was done (Benjamini and Hochberg, 1995). Moreover, a p-value under 0.01 and a threshold of 2-fold changes were applied. 8660 non redundant probe sets were differentially modulated (4212 for Cappelli, 7532 for Ofanto which 3084 in both cultivars). Using a QT-cluster analysis, the stress related genes were classified based on their expression profile in 52 clusters. Among them, 7 clusters have identified 1850 stress responsive genes characterized by significantly different stress expression profiles in Cappelli vs. Ofanto. Most of these genes are almost "drought insensitive" in Cappelli whereas they are slightly induced by drought in Ofanto. On the contrary, Cappelli is more responsive to heat stress than Ofanto. These genes might sustain the phenotypic differences in term of heat and dehydration tolerance. It is however remarkable that Ofanto and Cappelli show a huge differences in terms of molecular response to abiotic stress (Figure 6).

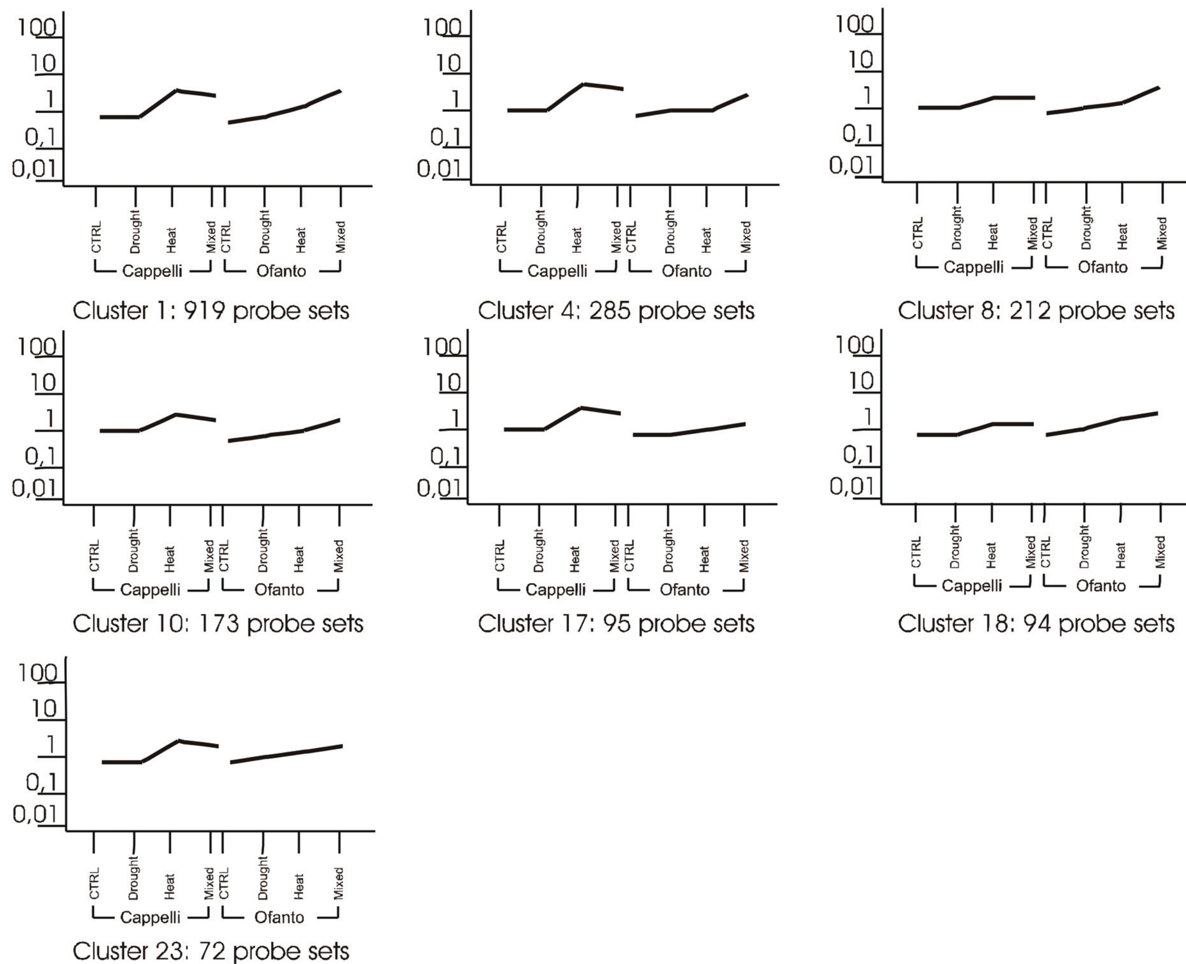

Figure 6 Graphs of the 1850 genes split in 7 genes clusters (1, 4, 8, 10, 17, 18 and 23). These genes show a clear expression trend in response to drought and heat stresses and a different expression profile between the two cultivars.

The *in silico* approach was applied to identify drought-induced gene transcripts with as large as possible difference in the expression profile between Ofanto and Cappelli. Genes that showed a differentially expression profile were found after comparing Ofanto and Cappelli samples. According to the biggest differences in fold change threshold of the Affymetrix dataset between the two cultivars or in the same cultivar at different drought-stress conditions and in according to the annotations associated to these genes, some drought response candidate genes were selected.

For annotation of the Affymetrix Probe Sets the informations came from PLEXdb (Plant Expression Database[1]). Sequence homology comparisons were used and performed initially against the TIGR Plant Transcript Assemblies database using Blastn program [2] in order to obtain Plant TA Accession and annotations. Sequences without

[1] http://www.plexdb.org/index.php
[2] http://blast.jcvi.org/euk-blast/plantta_blast.cgi

matches were compared to the other Blastn databases like DFCI Wheat Gene Index database[1] or NCBI[2] using the Blastn algorithm. In other cases was used Blastx UniProt database[3] and in some difficult cases was for high similar annotation searched in databases Blastx for Rice Genome[4] or *Arabidopsis thaliana* genome[5].

The TIGR Plant Transcript Assemblies database is in many ways complementary to other gene indexing databases and its clustering and high stringency approach offer a number of significant advantages over alternatives such as TIGR Wheat Gene Index database or GenBank. First, assembly provides a high confidence consensus representing each transcript and second, the produced consensus sequence is generally longer than its individual ESTs[6], providing a resource that can be used more effectively for functional annotation and for designing specific primers for its expression analysis. The sequences that are used to build the plant transcript assemblies (TAs) in the TIGR Plant Transcript Assemblies database are expressed transcripts collected from dbEST (ESTs) and the NCBI GenBank nucleotide database (full length and partial cDNAs). All plant species for which more than 1000 ESTs or cDNA sequences are available are included in this project. TAs are clustered and assembled to generate consensus sequences. Assembly criteria include a 50 bp minimum match, 95% minimum identity in the overlap region and 20 bp maximum unmatched overhangs.

3.3.2 Plant material

Seeds of the two wheat cultivars, Ofanto and Cappelli (for details, see Table 3; Figure 7), germinated in Petri dishes, were transferred to pots at Zadoks stage 0.7, i.e. when the coleoptile had emerged from the caryopsis (days after sowing are subsequently addressed as day of experiment, DOE). All plants of durum wheat were grown in square plastic pots with interior dimensions of 9,5x7x17cm (top diameter, bottom diameter, and height, respectively). Each replication required three pots, but

[1] http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi
[2] http://www.ncbi.nlm.nih.gov/
[3] http://www.uniprot.org/
[4] http://rice.plantbiology.msu.edu/index.shtml
[5] http://www.arabidopsis.org/index.jsp
[6] Expressed Sequence Tags (ESTs) are short (usually about 300-500 bp), single-pass sequence reads from mRNA (cDNA). Typically they are produced in large batches. They represent a snapshot of genes expressed in a given tissue and/or at a given developmental stage. They are tags (some coding, others not) of expression for a given cDNA library.

eight were prepared so that the most uniform three plants could be selected. The soil was composed of 80% turf and 20% vineyard soil and fertilizer was added. Each pot was filled with 500g of soil, then watered to excess, covered with a thin transparent plastic film and allowed to drain until reaching a constant weight, which was determinate as water holding capacity (the saturated weight, W_S) The pots contained drainage holes, but were supplemented with aluminum covers to prevent any water leakage. Seedlings were transplanting one to a pot and were grown in a growth chamber Sanyo, where temperatures were maintained at 20 ˚C during the day and 15 ˚C during the night. Photoperiod was 13 h (6 h – 19 h day) and 11 h (19 h – 6 h night) with average quantum flux density of PAR of 400 µmol quanta m^{-2} sec^{-1} during a 12 h day and humidity of 70% - 90% during the day and night respectively.

Pots were weighted daily, and control plants watered to 95% of relative soil water content (see below for determination of RSWC).

Table 3 “Ofanto” and “Cappelli” - basic characteristics.

Ofanto	Cappelli
cultivar was achieved from recent breeding program	is one of the first Italian cultivars
pedigree “Valnova” x “Appulo”	selection from the exotic landraces “Tunisia”
released 1990	released 1915
early heading	late heading
high harvest index (38.7*)	low harvest index (27.2*)
low stature (82 cm)	height stature (143 cm)
good stability of production in stress condition	is one of the first Italian cultivars

* (De Vita *et al*., 2007)

Figure 7 Picture of "Ofanto" (in the middle) and "Cappelli" (on the sides) in the field, CRA-GPG Fiorenzuola d'Arda, Italy

3.3.3 Relative soil water content, water stress treatments

Before planting, soil water holding capacity was determined by watering for each pot to excess, capping them with plastic lids then allowing them to drain until reaching a constant weight (the saturated weight, W_S). In addition, 500 g of soil from three extra pots was placed in a forced air drier at 150 °C for 24 hours, to estimate the oven-dry weight of soil that had been added to the pots (typically 366-369 g). Sum of the soil dry weight, the pot weight and the lids weight was used as the "dry weight" value (W_D). Relative soil water content (RSWC) was calculated as:

$$RSWC = ((\text{current pot weight} - W_D) / (W_S - W_D)) * 100$$

Four different treatments were studied with final target RSWC levels of 78/80, 49, 34/35 and 27/28%. The control plants were watered to 95% of relative soil water content and amount of the target weight (W_T) for adding water was calculated as:

$$W_T = ((W_S - W_D) * 0.95) + W_D$$

3.3.4 Vegetative measurements, samples collection

The leaves of the wheat plants were periodically measured for their quantity, length and width and after reaching required RSWC was collected one leaf from same last stage and immediately frozen in liquid nitrogen and kept at -80°C until use.

To obtain RNA from leaves subjected to water stress for use as targets in the qRT-PCR experiments, a slow water stress was applied by withholding water from plants for 23 days starting at the stage DC14-19 until approximately tillering DC22-29 (i.e. 39 days of DOE) (Figure 11). The uppermost fully expanded leaves were harvested at the same time to minimise any possible diurnal variation in gene expression, frozen immediately in liquid nitrogen and reserved for RNA isolation.

3.3.5 RNA extraction and cDNA synthesis

Total RNA was isolated from well-watered leaves and each of the stressed (78/80, 49, 34/35 and 27/28% RSWC) leaves using the TRI Reagent® Solution (Ambion; for details see Appendix 3). For each of the 78/80, 49, 34/35 and 27/28% stressed treatments were equal (3 x 100mg) quantities of leaves tissue combined to make a "stressed" pool. RNA concentration[1] and purity[2] were checked with a Beckman DU®640 spectrophotometer before and after DNAase treatment. The RNA concentration was determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA). The RNA samples with 260/280 wavelength ratio between 1.9 and 2.1 were used for cDNA synthesis. For total RNA cleanup was Qiagen RNA Cleanup Protocol used (see Appendix 4) and for removing the contaminating DNA, and to subsequently remove the DNase and divalent cations from the sample Ambion's DNA-*free*™ Kit was used (Appendix 5). After purification all samples were tested by PCR reaction of 20 ng of RNA template using the experimental primer pairs to detect the presence of residual DNA. For determining the integrity of RNA before cDNA synthesis the purified RNAs were loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to the manufacturer's instructions (for details see Appendix 6). The cDNA was synthesized with Standard Reverse Transcription Protocol (Promega,

[1] To determine the RNA concentration in µg/ml, was the A_{260} by the dilution factor and the extinction coefficient multiplied (1 A_{260} = 40 µg RNA/ml). **A_{260} × dilution factor × 40 = µg RNA/ml**.
[2] The A_{260}/A_{280} ratio of the RNA is an indication of its purity. The total RNA isolated with this procedure should have an A_{260}/A_{280} ratio of 1.8-2.2.

Appendix 7). For quantification of cDNA samples, Quibit fluorometerTM (Invitrogen) was used (Appendix 8).

3.3.6 RT-PCR analysis of drought-responsive genes

Primer designing and validation of qRT-PCR primers

Primer designing is very important step, because specificity of qRT-PCR is determined by their quality. It is therefore important to ensure high specificity of primer pairs for the coding sequence of the gene of interest and prevent formation of amplification products (primers must be unfavorable to the formation of dimers, hairpin loops, oligo duplexes, due to the presence of repeats and palindromes) that could limit the positive evolution of the reaction, contributing to the creation of secondary fluorescence, interfering with the signal given by the specific amplification product and giving Ct artificially early, providing an incorrect representation of the actual concentration the level of expression of tested gene. Because the experiment was carried out on cereal crop which is tetraploid hybrid (4n=28), for the production of specific primers was used a particular method. Common consequence of polyploidy in plants is to found in the genome sequences that are redundant or have small differences. Therefore the primers were designed on 3' UTR region (Untraslated Region) of the transcript, because it is a so-called hyper-variable region. This part is less subject of natural selection pressure and accumulates mutations.

Specific primer pairs were designed for all candidate drought-stress induced genes and for some reference genes on the basis sequences of their transcripts obtained from PLEXdb compared with the corresponding TC (Tentative Consensus) (for details see Appendix 9).

The complete set of primer pairs and their amplicon length, along with the corresponding sequence ID and their annotation, are listed in Table 4 and Table 6 for reference and target genes respectively. The specificity and uniqueness of the primers and the amplicons were first verified by PCR technique with subsequent electrophoresis on 2% agarose gel stained with Ethidium Bromide (EtBr), fluorescence under UV-light and sequencing of the PCR products by 3130*x*l genetic analyzer (Applied Biosystem, for the protocol see Appendix 10). Each PCR reaction was prepared using a 5 ng of cDNA derived from the RT reaction, 5 µl of 5× Green GoTaq Flexi Buffer (Promega), 1.5 µl of 25

mM $MgCl_2$, 0.25 µl of 5U Taq DNA polymerase, 0.5 µl of 10mM dNTP Mix and 400nM forward and reverse primers, in a total volume of 25 ml. The cycling conditions contained: 2 min at 95 °C, followed by 35 cycles of 95 °C for 45 sec, 59 °C for 30 sec and 72 °C for 45 sec with the final dissociation at 72 °C for 5 min.

Reference gene validation

Based on the fact, that well-known and frequently used housekeeping genes (HKGs) in wheat, whose protein products are involved in basic cellular processes has not stable and uniform expression across different tissues, developmental stages and combined effects of stress (Paolacci *et al*., 2009; Sharma and Kaur, 2009), some normal housekeeping and novel genes have been tested (Table 4).

For this proposal, we chose three HKGs genes (*TaRP15*, *TaRPII36* and *TaSnRK1*) with known primer sequences, which were used in expression analysis of transcription factors in *Triticum aestivum* (Stephenson *et al*., 2007). For another known reference genes as for cyclophilin and polyubiquitin (Aprile *et al*., 2009) new primers were designed from PLEXdb database (sequence ID: Ta.27454.2.S1_a_at and Ta.24299.1.S1_at, respectively). Primer designing for the last two novel reference genes (*TIM17* and *NADH-dehydrogenase*) correspond to PLEXdb probe set IDs: Ta.12727.1.S1_at and Ta.9617.1.S1_at, respectively was done for their stable behaving in microarray experiment. To search for a genes with stable level of expression in the condition used in microarray experiment the probe sets showing a "present" call in all hybridization examined based on the MAS 5.0 algorithm, were considered (Aprile *et al*., 2009). The uniqueness and annotation of the primers were confirmed by the database TIGR Plant Transcript Assembly BLAST Server. The selected HKGs genes are involved in biological process, like protein folding (cyclophilin), transport of proteins in and out of the cell (polyubiquitin), generation of mRNAs and most of the small nuclear RNAs (snRNAs) (RNA polymerase II (RNAPII) represented by TaRP15 and TaRPII36 primers) and transcriptional, metabolic, and developmental regulation in response to energy limitation and starvation of the carbon source (related protein kinase represented here as TaSnRK1). From the novel genes, (*NADH*) was annotated as NADH-ubiquinone oxidoreductase provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle, and (*TIM17*) as mitochondrial import inner

membrane translocase subunit of the TIM23 complex contributes to the architecture and function of the import channel.

To determine how the adoption of different reference genes can affect the normalization of the expression data for a gene of interest, the same 16 pooled cDNA samples included two different cultivars, developmental stages and drought stress (from 78/80% up to 27/28% RSWC plus the corresponding control plants) were used for qRT-PCR analyses of expression stability of reference genes. The main criterion was based on the principle that the expression ratio of hypothetical ideal control genes is identical in all samples, regardless of the experimental conditions or treatments. In this way, variation of the expression ratios of the samples reflects the fact that the gene is not constantly expressed, with increasing variation in ratio corresponding to decreasing expression stability. Genes with the lowest variation have the most stable expression and therefore would be selected as ideal reference genes and genes with the highest variation are removed. The expression stability of reference genes was analyzed by raw C_t values of each gene with 16 different samples. In this approach the mean value (Mv), standard deviation (SD), standard error (SE), coefficient of variation (CV) and delta C_t (ΔC_t) were calculated. These data sets were used to evaluate the genes expression stability.

Table 4 Reference genes designed on the basis of stable expression outputs of Affymetrix or obtained from literature, which were tested for their stable expression in tissue under different drought-stress condition.

Primer name Probe Set ID/TC	**Best match annotation** Accession	**Score**	**Blastn e-value**	**Ampl. length (bp)**	**Primer sequence (forward/reverse)**
Cyclophilin Ta.27454.2.S1_a_at	Cyclophilin [Triticum aestivum (Wheat)] TIGR|TA60203_4565	2693	4.0e-125	58	5'-CCGATGAGAACTTCAAGCTCAA-3' 5'-GCATTGGCCATGGAAAGGT-3'
NADH-dehydrogenase Ta.9617.1.S1_at	Putative NADH dehydrogenase [Oryza sativa (japonica cultivar-group)] NADH-ubiquinone oxidoreductase 23 kDa subunit, mitochondrial protein TIGR| TA66367_4565	4597	2.4e-201	55	5'-CCCAGCCCAGGCAATTACT-3' 5'-TCGGCGACTGCCATCTTC-3'
Polyubiquitin Ta.24299.1.S1_at Aprile *et al*., 2009	Ubiquitin C variant [Homo sapiens (Human)] TIGR/PTA| TA51676_4565	2977	1.9e-137	67	5'-TTTGCCGGTTGATTGAAGTG-3' 5'-TCCAAGTACAGGTGACAGCTGACT-3'
TaRP15 Stephenson *et al.*, 2007	T. aestivum Putative DNA-directed RNA polymerase II subunit 15 kDa related cluster TIGR| CA673054	110	1.00000	56	5'-GCACACGTGCTTTGCAGATAAG-3' 5'-GCCCTCAAGCTCAACCATAACT-3'
TaRPII36 Stephenson *et al.*, 2007	(T. aestivum RNA polymerase II 36 kDa subunit) TIGR| BG908025	135	1.00000	84	5'-ACGTATTAACCAAGAACTCATGGAGAC-3' 5'-TCAAATACTTTTGTAGGGCTGCTCTC-3'
TaSnRK1 Stephenson *et al.*, 2007	SNF1-related protein kinase [Hordeum vulgare (Barley)] TIGR| TA71609_4565	130	1.00000	97	5'-GCTTCGGTGTGGAGTCTGCTAT-3' 5'-TCCCTTGTTTTGTAGAGCTGAATTTC-3'
TIM17 Ta.12727.1.S1_at	Protein At2g42210 [*Arabidopsis* thaliana (Mouse-ear cress)] Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein TIGR| TA66676_4565	4087	2.7e-178	81	5'-GATGCACCTAGTTCCTTTGCAA-3' 5'-GCAAGATATTTATTCCAGGTCTGTTG-3'

qRT-PCR conditions

Quantitative real time polymerase chain reaction was performed with SYBR Green fluorescence detection in a qPCR thermal cycler (ABI PRISM 7300, Applied Biosystems). Preliminary qRT-PCR assays carried out on a pool of available cDNAs using six different primer concentrations ranging from 250 to 1000 nM showed that the optimal primer concentration was 1 μM (as suggested by Qiagen protocol), because it generated the lowest C_t value, a sharp peak by melting curve analysis and absence of non-specific products or primer-dimer artefacts. The specificity of the amplicons was checked by gel electrophoresis and sequencing of the PCR products in order to confirm that its sequence corresponded to the target gene (see Appendix 9). Five-point standard curves of a 5-fold dilution series (1:1-1:625) from pooled cDNA were used for PCR efficiency calculation of each primer pair (Figure 8 and Figure 9). The PCR efficiency (E) is given by the equation $E=10^{(-1/slope)}-1$, where slope is linear regression model fitted over log-transformed data of the input cDNA concentration versus C_T values according to the linear equation y=slope*log(x)+b. If is slope equal to -3.32[1] efficiency becomes 1 (100%).

Each reaction was prepared using 3 μl from a 2 ng/μl dilution of cDNA derived from the RT reaction, 12.5 μl of SYBR Green PCR Master Mix (Qiagen), 1 μM forward and reverse primers, in a total volume of 25 ml. For each primer pairs two no template controls were run to detect contamination and dimer formation. The cycling conditions contained: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min with the final dissociation at 95 °C for 15 sec, 60 °C for 30 sec and 95 °C for 15 sec. Two wheat genes: *cyclophilin* and mitochondrial import inner membrane translocase subunit *TIM17* were used as internal reference genes for calculation of relative transcript levels of the genes under study. Three biological replicates were used for quantification analysis and three technical replicates were analyzed for each biological replicate. Gene expression levels were recorded as C_t values, which are inversely related to the initial DNA concentration. C_t values were defined as the number of cycles required for normalized fluorescence (Rn) to reach as manually set threshold of 20% total fluorescence. The ABI PRISM 7300 Sequence Detection System software calculates the ΔRn using the equation ΔRn = (ΔRn+) – (ΔRn-), where Rn+ is the fluorescence signal of

[1] Slope -3.3 to -3.4 is considered to be optimal, lower value (<-3.3) means "too efficient" PCR, while higher value (>-3.4) points on the low PCR efficiency.

the product at any given time and Rn- is the fluorescence signal of the baseline emission during cycles 6-13. Melting curve analysis was performed to evaluate the presence of non-specific PCR products and primer dimers. Moreover, we consider only C_t technical repetitions differing by less than one cycle. Comparative C_t method calculation steps to calculate the fold changes (FC) consist of the following three steps: 1) normalization to endogenous control by comparison of target gene and endogenous control $\Delta C_t = C_{t\ target\ gene} - C_{t\ endogenous\ gene}$; 2) normalization to calibrator sample $\Delta C_{t\ sample} - \Delta Ct_{calibrator} = \Delta\Delta C_t$; 3) using the formula $2^{-\Delta\Delta Ct}$. The CT data were expressed as average of tree experimental replicates. As calibrator was used in all cases Ofanto irrigated (95% RSWC).

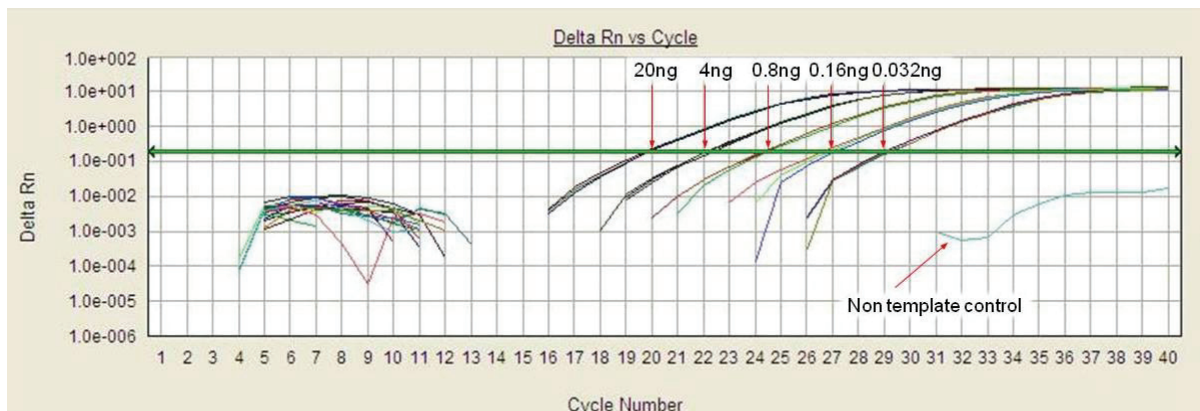

Figure 8 The *TIM17* gene was amplified from a 5-fold dilution series (1:1-1:625) from pooled cDNA. The green line indicates threshold and C_t value for each series of absolute quantification.

Figure 9 Standard curve of the *TIM17* gene from previous picture above, slope -3.310522 indicates 99.5% efficiency.

Sample preparation and target gene validation

Total RNA was isolated from 48 plant leaves providing 16 different stressed samples. DNase digested and reverse transcribed. Two-step qRT-PCR protocol, reverse transcription and PCR-mediated cDNA amplification was carried out by subsequent steps in separate tubes. The two-step protocol was prefered because it would reduce

unwanted dimer formation between primers when SYBR Green is used as a detection dye and also was taken into account, that double measurements of concentration on the level of total RNA and cDNA will have had great impact on the precision of the following analysis. The RT reaction was primed by oligo-(dT), rather than using random-sequence primers which anneal preferentially to abundant mRNA species. This choice provides a more accurate overview of gene expression. PCR primer pairs for selected reference and target candidate genes were designed and preliminarily tested in PCR and qRT-PCR reactions from a pool of all available cDNAs (see methods). In order to avoid unreliable amplification of splice variants and assure uniform RT efficiencies, typically primer pairs were designed to target an amplicon located within the 3' end region of the transcript of interest. For each biological replicate the same cDNA pool was used for qRT-PCR amplification of the 20 analysed genes. Moreover, qRT-PCR analysis of every gene was performed in triplicate for each of the 16 cDNA pools, along with no template control. The 20 primer pairs used to amplify the candidate target genes generated single amplicons of the expected size from the various cDNA pools, as shown by the presence of single bands in agarose gel electrophoresis and by single-peak melting curves of the qRT-PCR. A detailed analysis of the dissociation curves confirmed the absence of primer dimers and of other products resulting from non-specific amplification (for more information see Appendix 11). A more stringent test of PCR specificity was performed by sequencing the amplification products of each of the 20 candidate target genes. Always the sequence of the PCR product matched that of the target cDNA, thereby confirming the exquisite PCR specificity of the developed primer pairs.

3.4. Results

3.4.1. Identification of genes differentially expressed in Ofanto and Cappelli in response to drought stress based on microarray data analysis

Differential expressed genes showing different stress expression profiles were found in Cappelli vs. Ofanto samples. According to the fold change threshold of the Affymetrix dataset and the annotated activity, seventeen drought response genes markers were selected. The list includes genes involved in stress perception, plant protection, adaptation of essential physiological processes (photosynthesis) in response

to water, salt or temperature-stress, as well as transcription factors involved in the regulation of the plant response to stress. The selected genes are listed in Table 6 and their expression profiles as detected in the microarray data set are illustrated in Figure 10. Of this 17 genes, 13 (76,5%) were greater than two-fold up-regulated under water stress in Ofanto and 5 (29%) in Cappelli, while 1 (6%) was greater than two-fold down-regulated under water stress in Ofanto and 3 (18%) in Cappelli; the remaining were not significantly (two-fold) up- or down-regulated (Figure 10).

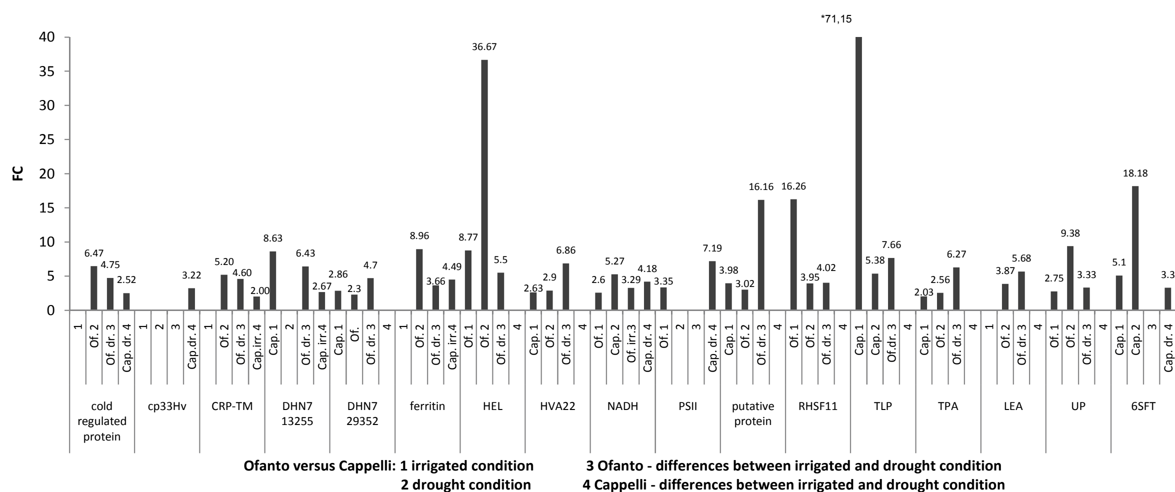

Figure 10 Data from Affymetrix hybridization experiments expressed as fold change (FC). The results showed differences in gene expression between Ofanto and Cappelli cultivars in non stressed/irrigated (line 1) and stressed/drought (line 2) condition in response to increasing drought stress. Significant variation in expression after exposure to drought stress has been detected in Ofanto (line 3) and Cappelli (line 4) cultivars after comparison with their control plants. FC below 2 are not reported because not significant. *the peak was corrected for comparison to the others.

3.4.2. Physiological parameters of the water-stressed plants

Water was withheld from wheat plants for a 23-day period (to simulate gradual development of a field-like water stress) beginning at the stage DC13. The leaves of the wheat plants were periodically measured for their quantity, length and width. Early, during the stress process, plants were phenotypically indistinguishable from well-watered plants. Until ninth day of this period cv. Ofanto showed slightly higher soil water consumption. This consumption rate completely changed after reaching 65% RSWC. Since this time Ofanto showed lower soil water consumption (Figure 11). However, plants mid-way through the stress period displayed leaf wilting, leaf curling and reduced leaf length and width. Over the 23-day period of withholding, when relative soil water content decreased to approximately 27/28% (for comparison see Figure 13)

this difference in water consumption between Ofanto and Cappelli reached approximately 8% and Ofanto significantly decreased even in the number of the tillers (Figure 12). Although from the Figure 11 results showed that Cappelli consumes water more quickly, after comparison of the water needs for the creation of one gram of biomass Ofanto reported from 14% higher water use in irrigated plants to 24% water use in drought plants in comparison with Cappelli. Cappelli demonstrated in all cases (stressed and non-stressed conditions) higher biomass production and after comparison its water consumption was confirmed its superior ability for "water use efficiency[1]" (WUE) (Table 5).

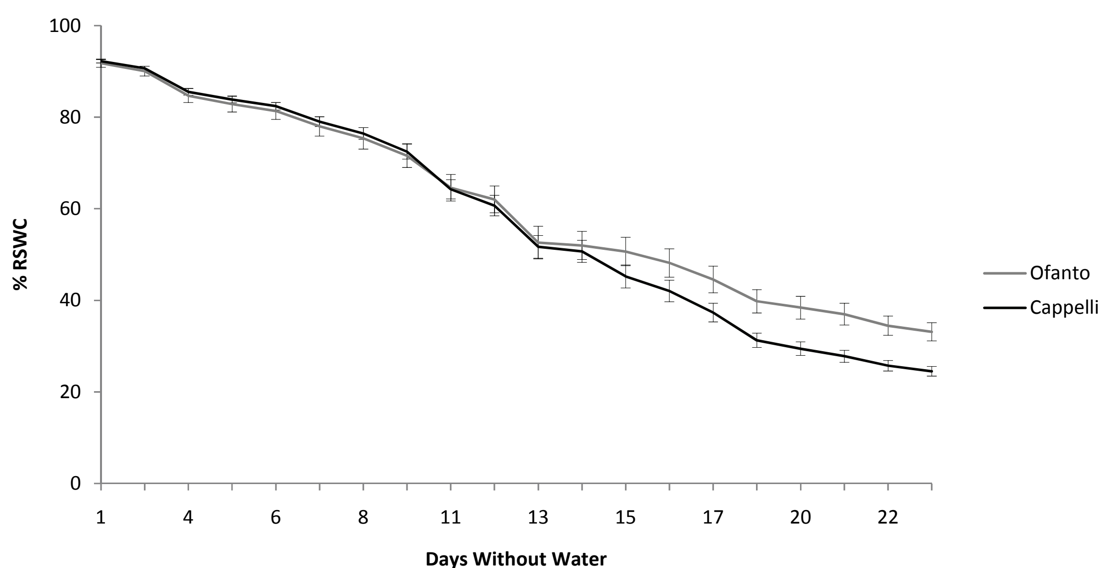

Figure 11 Wheat plants at seedling growth were subjected to water deficit stress treatment for 23 days and RSWC (%) was measured each day. The measurement is recorded together with standard error (SE) of the estimate of the mean.

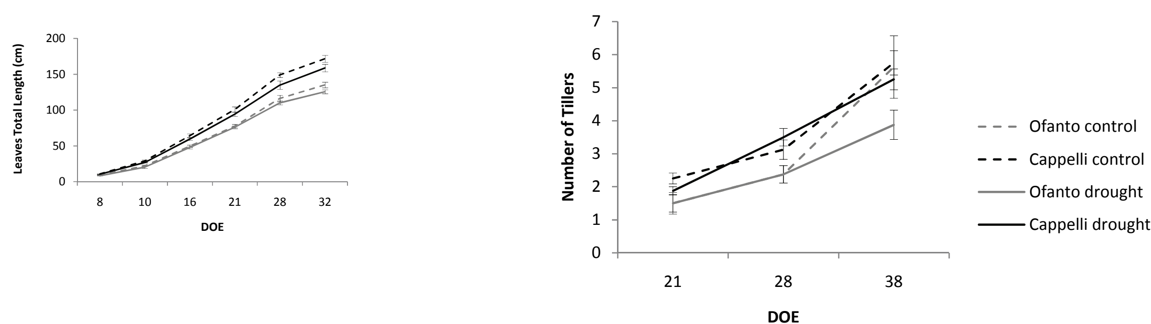

Figure 12 Plant measurements during 39 days of experiment (DOE) in control plants (95% RSWC) and plants, where a slow water stress was applied by withholding water for 23 days starting at the stage DC14-19 until approximately tillering DC22-29 (drought). The values shown here represent the mean value of the 8 plants in the same treatment, together with the standard error (SE).

[1] Water use efficiency (WUE) is defined as the ratio of total dry biomass to total water consumed.

Figure 13 Ofanto control (95% RSWC);
Ofanto drought (28% RSWC); Cappelli control (95% RSWC); Cappelli drought (27% RSWC); respectively.

Table 5 Morphological and physiological data measured between two cultivars Ofanto and Cappelli were performed on control plants and plants following of drought stress effect together with standard error.

	Control Plants (95% RSWC)		Stressed Plants (27/28% RSWC)	
	Ofanto	Cappelli	Ofanto	Cappelli
Total fresh biomass (g)	10.22 ± 2	14.1 ± 0.83	3.72 ± 0.23	7.69 ± 0.6
Total dry biomass (g)	1.08 ± 0.2	1.37 ± 0.05	0.64 ± 0.05	1.18 ± 0.04
Additional tillers/main tiller	11.9 ± 1.8	13 ± 1.1	9.4 ± 1.5	6.9 ± 0.4
Total water consumption (g)	551 ± 89	630 ± 19	261 ± 16	397 ± 13
Water consumption (g)/dry biomass (g)	531 ± 28	463 ± 10	420 ± 30	337 ± 8
WUE –dry biomass (mg)/water consumption (g)	1.92 ± 0.09	2.17 ± 0.05	2.45 ± 0.15	2.98 ± 0.07

3.4.3. Designed primers for drought-induced genes

17 primer pairs in the Table 6 below were designed from the available probe sets as was described in methods above. In addition another six promising genes, which are involved in tolerance of water- and salt-stress (Δ-1-pyrroline-5-carboxylate synthetase - *P5CS*) (Silva-Ortega *et al.*, 2008; Székely *et al.*, 2008; Wang *et al.*, 2011), stomatal guard cells and vascular tissue (OPEN STOMATA 1 - *OST1*) (Belin *et al.*, 2006; Mustilli *et al.*, 2002), cell cycle regulation and plant root growth (*MYB59*) (Mu *et al.*, 2009; Du *et al.*, 2009), transpiration efficiency regulation (*ERECTA*) (Masle *et al.*, 2005), chlorophyll (Chl)-synthesis pathway and plastid-to-nucleus signaling (a key three-component catalytic enzyme *Mg-chelatase*) (Shen *et al.*, 2006a; Shang *et al.*, 2010) and in encoding of major isoenzymes of chloroplast fructose bisphosphatase (*TaFBPase_1*) (Xue *et al.*, 2008) were chosen (Table 7). For some those genes were primer pairs from Tentative

Consensus (TC) of DFCI Wheat Gene Index database, or Probe Set sequences (Ta.-) from PLEXdb designed. Some primers from literature were also used.

Table 6 The most regulated transcripts from the drought/heat – stressed tissue vs. well watered tissue microarray comparison. Table with designed primers and annotation obtained from TIGR Plant Transcript Assembly (TIGR), GenBank (gb) and Wheat Gene Index databases (WGI).

Primer name Probe Set ID[1]	**Query length**	**Best match annotation** Accession	**Score**	**Blastn e-value**	**Ampl. length (bp)**	**Primer sequence (forward/reverse)**
cold.r.prot Ta.13183.1.S1_x_at	990	Cold regulated protein [*Triticum aestivum* (Wheat)] TIGR|TA69108_4565	3249	2.5e-140	83	5'-TTTTGACACTCGGCTCTGTG-3´ 5'-GCACGCTACAGAACAGACCA-3´
Cp33HV Ta.18434.1.S1_at	1580	Cp33Hv [*Hordeum vulgare* (Barley)] TIGR|TA37056_4513	2283	3.5e-160	80	5'-GTTCAAAAATTCTGCCGTTCCA-3´ 5'-CGTGGCTGAAATCCCACTTC-3´
CRP-TM Ta.13183.1.S1_s_at	990	Cold regulated protein [*Triticum aestivum* (Wheat)] TIGR|TA69108_4565	3249	2.5e-140	105	5'-GATTTTGACACTCGGCTCTGT-3' 5'-TCGTTGCCATGCGTATACAGG-3'
DHN7 13255 Ta.13255.1.S1_at	850	Dehydrin related cluster TIGR|TA63835_4565	3723	9.1e-162	81	5'-GCCACCTCTTGCAGAATAATAAGAT-3' 5'-ATTCAAGTCCACAAAACTCAGAACTC-3'
DHN7 29352 Ta.29352.1.S1_x_at	880	Dehydrin 5 [*Hordeum vulgare* (Barley)] TIGR|TA63834_4565	3879	7.7e-169	80	5'CTTCCCGAAATAAAGTGAGCTAGCT-3' 5'-AAGCAAGCAACCCAAATTCAA-3'
ferritin Ta.681.2.S1_a_at	636	Ferritin [*Triticum monococcum* (Einkorn wheat) (Small spelt)] TIGR|TA57609_4565	1785	2.2e-74	69	´5-TCGACCAGATGCTGCTTGAG-3' ´5-CATCCTCCATTGCCCGTAGT-3'
HEL TaAffx.100436.1.S1_at	758	- TIGR|TA97230_4565	3690	2.9e-160	96	5'-GGATTGTCAGGATGGCTATGTG-3' 5'-TCAGTAAGACCAACACCATTTTTAGG-3'
HVA22 Ta.16038.1.S1_at	883	HVA22 protein related cluster TIGR|DR739435	3009	8.5e-165	108	5'-TGGAGCAGCAACCAGAGATCC-3' 5'-GCACCACAAGCTAAAATCTCAAA-3'
LEA Ta.5913.1.S1_at	889	Group 3 LEA protein [*Oryza sativa* (Rice)] TIGR|TA57605_4565	4183	1.4e-182	100	5'-ATCACAAAGAGCCGCCACTA-3' 5'-TCGGACACACCACAGGAAT-3'
NADH TaAffx.112816.1.S1_at	569	NAD(P)H-quinone oxidoreductase chain 2, chloroplast (EC 1.6.5.-) (NAD(P)H dehydrogenase, chain 2) related cluster TIGR|CA620653	2332	8.0e-99	100	5'-CCCCCTAGAGTAGCTGTTAATACGAA-3' 5'-GTTCAACTTTATGTATTCCTCTATCCGTAGA-3'
PSII Ta.28750.2.A1_x_at	533	Photosystem II 10 kDa polypeptide, chloroplast precursor [*Hordeum vulgare* (Barley)] TIGR|TA53869_4565	2111	6.0e-89	80	5'-GCTGTTTCTCTGGGCAATCAC-3' 5'-AAGCCAAGGCACTAGTATTGTAGACA-3'
putative protein Ta.29464.1.A1_at	635	Hypothetical protein P0042A10.38-1 related cluster TIGR|CK211571	2575	4.7e-110	78	5'-GTCAATTTTGTTCGGCTCATCTT-3' 5'-CGATTTGCATCAACCAACG-3'
RHSF11 TaAffx.34778.1.S1_at	963	Putative heat shock transcription factor 8 related cluster TIGR|BE418746	2690	4.2e-115	106	5'-GCCGTTCCCCTTCTCTAATC-3' 5'-CATAAACTCCAAAACATCACACG-3'
TLP Ta.25053.1.S1_at	960	Thaumatin-like protein [*Triticum aestivum* (Wheat)] TIGR|TA65743_4565	4519	8.7e-198	81	5'-CGGCGGCGACACCTATT-3' 5'-TTCCCCTTGAAGAACTTCGAGTA-3'
TPA Ta.3145.1.S1_at	772	- TIGR|TA68643_4565 *Triticum aestivum* cDNA, clone: SET1_O05, cultivar: Chinese Spring gb|AK332714.1 - WGI|TC397613	3253 601 3253	1.6e-140 4e-171 9.6e-142	71	5'-CAATAAGAATGAACTTACGAGTATGCAGTA-3' 5'-TTTCGTGGGAAAATCGACAGA-3'
UP Ta.28273.1.S1_x_at	474	Hypothetical protein [*Arabidopsis thaliana* (Mouse-ear cress)] TIGR|TA64737_4565	1897	4.1e-79	58	5'-GGAGAGCGGCAGGGACTAC-3' 5'-CACGGCATGAGGTGCTACAC-3'
6-SFT Ta.2789.2.S1_at	2553	Sucrose:fructan 6-fructosyltransferase [*Triticum aestivum* (wheat)] TIGR|TA50723_4565	10392	0	75	5'-CCAAGGAAGGCCAAGACATATG-3' 5'-GACCCAAGGATAGATGTTTCAATTC-3'

[1] The ends of these probes have the following meanings: **_at -** all probes are unique to this exemplar, **_s_at -** all probes are perfect match to another exemplar, **_x_at -** at least one probe is perfect match to another exemplar, **_a_at -** at least one probe is a perfect match to another exemplar that is considered to be a different gene.

Table 7 Sequences of the specific primers for drought-related genes and source of sequence/publication.

Primer	Nucleotide sequence forward	Nucleotide sequence reverse	Lenght (bp)	Source
ERECTA	5'-TTCTGCGACCGTTCTTGGA-3'	5'-GACATTGCTACCGACATCCTTACA-3'	69	TC256229
Gene 59	5'-CATCAAGCTGCACAACATGCT-3'	5'-TTCCAGACGTTCTTGATCTCGTT-3'	93	TC142211
Mg-chelatase	5'-TCTACTACTCGGAGGTTGAAGACAAGA-3'	5'-GGAACTGGTTGATCAGTGAAATCAG-3'	115	TC236115
OST-1	5'-CCACCATACCACCTGCTTGTT-3'	5'-TGATTCAAGGTCATCCATGTC-3'	86	Ta.991.1.S1_at
P5CS	5'-AGGTCATAACCAGTGTGATTCC-3'	5'-TTGCTTTTCACAAGGCCAATA-3'	64	Ta.7091.1.s1_at
TaFBPase _1	5'-ACACCGTGACTCTGGAGGAAGT-3'	5'-AAGAGAAGGGTCAAGCGTGAAG-3'	139	Xue *et al.*, 2008

3.4.4. Selection of the best candidate reference genes for qRT-PCR analyses and their expression stability

To select the most stable gene with expression stability was compared CV values and the C_t differences (ΔC_t= $C_t$max-$C_t$min) obtained by the 7 primer pairs for each of the 16 samples (see Appendix 11, Table 13,). The CV of the C_t values gives a straightforward indication of the expression stability of a particular gene. The genes with the lowest CV values and also the lowest values of C_t range (ΔC_t) were chosen. Table 13 and also Figure 14 show that the most stable genes with lowest CV values and ΔC_t were corresponding to the probe set Ta.12727.1.S1_at, a probe set annotated as mitochondrial import inner membrane translocase TIM17 (ΔC_t 1.47; CV 0.021), Ta.9617.1.S1_at, a probe set annotated as NADH-dehydrogenase (ΔC_t 2.41; CV 0.029) and to the cyclophilin (ΔC_t 2.84; CV 0.033). For this reason *TIM17* was chose as representative and used for further comparison with the other candidate target genes in all series except 34/35% RSWC series, where a ΔC_t difference was higher than 1 observed, for this group of samples was *cyclophilin*, with lower ΔC_t (0.62) as reference gene used (for details see Table 13). For the other genes included those for which expression was considered relatively stable under all condition were observed higher changes significantly under some treatment.

As discussed in the previous part, it was evident that none of the 7 selected candidate reference genes showed a constant expression level under all tested conditions (cultivars, drought stress and developmental stages) in wheat. Hence, as expected, it was not possible to find an all purpose reference gene, and was necessary to select the most suitable one for normalizing gene expression considering the peculiar and specific experimental conditions for each series of RSWC treatment. Anyway as most stable gene across tissue, development and stress treatment has been identified in durum wheat *TIM17*. This provides a new reference gene for future studies in similar durum wheat experiment. The high expression stability of a gene indicates that the use of a single internal control is appropriate, but some studies require the normalization

based on two or more stable internal control genes. It confirms necessity of using adequate reference genes separately, depending on the specific experiment, the growth phase and type of treatment.

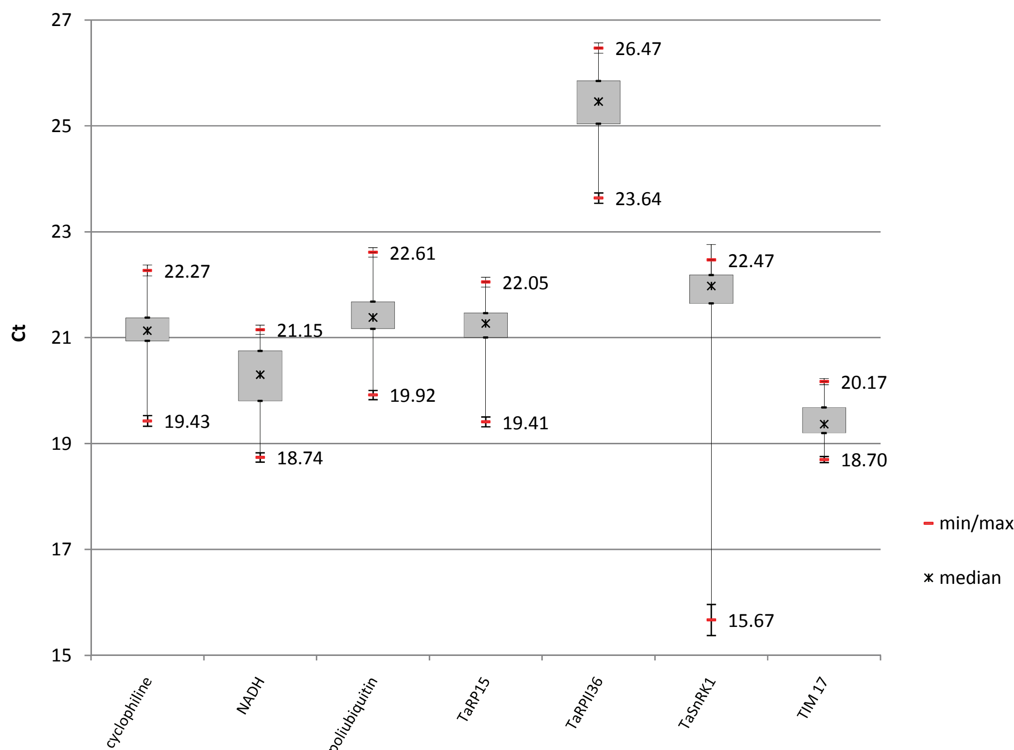

Figure 14 The expression stability of reference genes was analyzed by raw C_t values of each gene with 16 different samples (in three repetitions). The variation of house-keeping genes is show in graph by median, interquartile range (grey boxes) and max/min Ct value with standard error.

3.4.5. Expression levels of the selected candidate target genes

From the origin 22 genes of interest based on the results of the PCR and qRT-PCR standard validation experiment 15 couple of primers from microarray plus other 5 primers for other drought-related candidates gene detection were chosen to test their expression level on Ofanto and Cappelli by qRT-PCR analysis. The others were excluded for their non-specific behavior. Only two-fold and more changes in gene expression were recorded. Our proposal was to validate the drought related sequences in plants at tillering stage. These primers were tested by qRT-PCR on the RNA extracted from the last developed leaf (main tiller) of the parents during a water withholding experiment. The drought stress levels were from the lightest 78/80%, 49%, 34/35% to the strongest

27/28% of Relative Soil Water Content (RSWC). As expected, most significant differences in gene expression of target genes were observed under the strongest stress effect (27/28% of RSWC). Because stable gene expression with such differences is crucial requirement to map an expression-QTL, our attention was consequently focused on the strongest stress treatment as shown bellow (Figure 15). Moreover also other expression differences within cultivars due to water withholding were recorded. Of the 20 drought-induced genes, 9 (45%) were greater than two-fold up-regulated under water stress in Ofanto and 2 (10%) in Cappelli (see Figure 15; "drought plants 27/28% RSWC"); the remaining were not significantly (two-fold) up- or down-regulated between two cultivars under drought-stress. Overall, after comparison of gene expression as a result of stress in both cultivars, where is 12 of 20 genes expressed, Ofanto showed much higher genes expression, than Cappelli and this also corresponded with results of previous Affymetrix experiment.

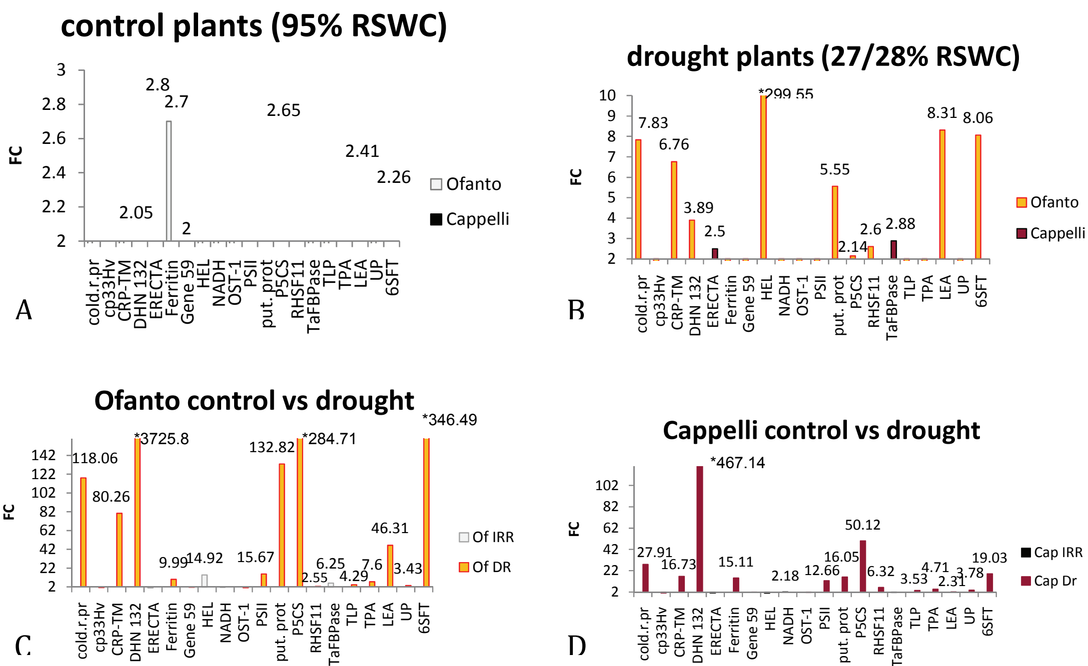

Figure 15 Results related to the Fold Change (FC) qRT-PCR between Ofanto (Of) and Cappelli (Cap) in IRR-irrigated 95%RSWC (figure A) and DR-drought 28/27% (figure B). Ofanto control (95% RSWC) vs drought (27/28% RSWC) in figure C; Cappelli control (95% RSWC) vs drought (27/28% RSWC) in figure D. *this peaks were corrected for comparison to the others.

Table 8 Summary table: comparison drought induced genes (FC ≥ 2, labeled here as plus) in an independent microarray experiment vs. qRT-PCR experiment by using the same genotypes.

	Microarray		qRT-PCR		
Ofanto	Drought	Irrigated	Drought	Irrigated	Match
Cold r. prot.	+		+		Yes
Cp33Hv					
CRP-TM	+		+		Yes
DHN132	+		+		Yes
Ferritin	+		+		Yes
HEL	+			+	No
NADH		+			
PSII			+		
Putative protein	+		+		Yes
RHSF11	+		+		Yes
TLP	+		+		Yes
TPA	+		+		Yes
LEA	+		+		Yes
UP	+		+		Yes
6SFT			+		
Cappelli	Drought	Irrigated	Drought	Irrigated	Match
Cold r. prot.	+		+		Yes
Cp33Hv	+				
CRP-TM		+	+		No
DHN132		+	+		No
Ferritin		+	+		No
HEL					
NADH	+			+	No
PSII	+		+		Yes
Putative protein			+		
RHSF11			+		
TLP			+		
TPA			+		
LEA			+		
UP			+		
6SFT	+		+		Yes

3.5 Discussion

One of the aims of the present research is to identify genes involved in the molecular response to drought for better understanding the mechanisms that regulate the plant tolerance to abiotic stress in order to improve yield stability. This importance is increasing by the impact of climate change on agricultural production. Since apparition of high-throughput sequencing tools[1] and bioinformatics allowing a whole-genome analysis approach to gene expression becomes this task easier than in the past. Comparative mapping studies between rice, maize, sorghum, barley and wheat have pioneered the field of the plant comparative genomics a decade ago. They showed that the linear order (colinearity) of genetics markers and genes is very well conserved opening the way to accelerate the map-based cloning and defining rice as a model for grasses. In recent years, *Brachypodium dystachion* has also emerged as a new model

[1] Sequences derived from "next generation" sequencing platforms (NGS; beginning in 2009), including Roche 454, Illumina, Applied Biosystems SOLiD, and Helicos Biosciences HeliScope, provides data, which should be submitted to the Short Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra).

genome for *Triticeae* (Feuillet *et al*., 2009). The first sequence assembly indicating a Brachypodium genome size as relatively small (~300 Mbp) (Vogel *et al*., 2010). This opens the possibility to study genes in species where is still not known complete genome by using available information from other species which are closely related. It is likely that the annotation of the genes derived from other species can be useful for the identification and isolation of a target region but candidate genes have to be carefully defined and worked with on a case-by-case basis. There have been a number of other studies using microarrays to examine the effects of dehydration stress in plants causing changes in mRNA expression and many genes have been previously reported as differentially regulated under drought (Matsui *et al*., 2008; Talamè *et al*., 2007; Cattivelli *et al*., 2008). Comparative analysis of some microarray studies across species (arabidopsis, wheat, maize, rice) and experiments highlight that the conservation of the molecular response to dehydration is generally low despite the presence of common regulatory mechanisms (Aprile *et al*., 2009). Other microarray comparative study of the expression changes in leaves of barley plants subjected to slow or rapid drying showed only a low percentage of up- or down-regulated transcripts (~10%) with similar expression profile regardless of the dynamics of the water stress treatment. Irrespective to this low number of transcripts with similar expression changes between those two treatments a sizeable portion of these transcripts shared a common expression trend under the different drought treatment conditions (Talamè *et al*., 2007). This study indicated changes in expression profiles that varied considerably according to the dynamics of the dehydration treatment (i.e. shock-like versus gradual stress). The gradual dehydration of leaves during slow drought imposition revealed a lower number of differentially regulated transcripts, than was observed in sudden and more severe dehydration. Also in De Leonardis *et al*. (2007) study of durum wheat up-regulated genes tested for their expression in plants at different stages of development and exposed to different dehydration conditions, was based on the results showed that a general frame of the molecular response to water stress can not be considered so generic. This study indicated that the plant molecular response to dehydration changes continuously resulting from an integration of a series of internal and external stimuli.

In our study the expression profile of 15 genes has been verified in an independent experiment carried out at tillering stage using the same genotypes used for the microarray experiment at booting stage. From 11 drought-induced genes in Ofanto

microarray experiment, 10 genes confirmed same behaving in RT-PCR experiment except for "High Expression Level" gene (*HEL*) that showed an opposite expression trend (down regulation). *PSII* and *6SFT* genes were significantly up-regulated under water stress in qRT-PCR experiment, while in Affymetrix experiment was this expression not observed. In microarray experiment of Cappelli 5 genes (*cold. r. prot.*, *cp33Hv*, *NADH*, *PSII* and *6SFT*) were greater than two-fold up-regulated under water stress, after comparison by qRT-PCR stability of 3 genes (*cold. r. prot.*, *PSII* and *6SFT*) was confirmed, while the other ones (*cp33Hv* and *NADH*) were not significantly regulated. Another nine genes were up-regulated under water stress in Cappelli qRT-PCR experiment, while not in Affymetrix experiment. This fact confirms the stability of gene expression largely drought-induced genes across developmental stages in Ofanto, while Cappelli demonstrated significant changes in strategy of gene expression. This can explain bigger differences between microarray gene expressions which showed greater than two-fold up-regulation under water stress for 11 genes in Ofanto and for 5 genes in Cappelli, whereas results from qRT-PCR experiment showed more extensive changes, namely 12 genes were up-regulated in Ofanto and 12 in Cappelli. Otherwise after comparison at expressions level between two cultivars under drought stress, some changes in intensity of gene expression are visible and provide changes in the final gene expression between those two cultivars (9 genes were more than two-fold up-regulated in Ofanto and only 2 genes in Cappelli).

The durum wheat genotypes used in this work showed different reactions to the water stress treatment when grown in soils with the same amount of available water. The cultivar Ofanto was previously characterized as tolerant to water stress based on antioxidant defense system, pigment composition, and photosynthetic efficiency in drought conditions (Loggini *et al.*, 1999). Furthermore, Ofanto was showed to be better adapted to water stress compared to the other sensitive cultivars in terms of relative water content, cell turgor and lipid-to-protein ratio in thylakoids (Quartacci *et al.*, 1995). Ofanto is a high yielding relatively recent cultivar derived from Appulo x Valnova, released in 1990, representative of modern cv. The cultivar Cappelli confirmed in similar experiment higher amount of fresh biomass during dehydration, then observed in Ofanto and also sum of leaf lengths of Cappelli was slightly, no significantly, but constantly higher than the sum of leaf lengths of Ofanto both in watered and droughted plants. In contrary opposite results were obtained in Ofanto, which had consumed on

average from 21% to 7% more water than Cappelli under well watered and drought condition, respectively (Rizza *et al*., unpublished). The main results of both works obtained from phenotype observation confirmed that WUE was higher in both cases in Cappelli than in Ofanto. Cappelli is an old, low yielding cultivar, released in 1915, derived from the exotic landrace Tunisia which was not improved over the last century but conserved an higher WUE, a important agronomic character.

Figure 15 reports the expression of the transcripts by qRT-PCR. It should be noted that for those genes that responded rather similarly to a decrease in water content irrespective of the dynamics of the dehydration or stage of development may actually provide an easier, faster, and more effective way to identify and characterize functionally different alleles involved in the adaptive response to drought. For this, a critical analysis, available data from existing transcriptome profiling experiments and from protein and metabolite profiling studies conducted under various dehydration regimes may reveal additional information useful to identify candidate genes and processes whose manipulation may improve drought tolerance. Occurrence of up-regulated rather than 299 FC under drought stress condition in Ofanto for HEL transcript encoding for unknown protein involved in the response to abiotic stimulus and stress was considered as most likely candidate gene to identify the correspective expression QTL in the Ofanto x Cappelli segregating population (Chapter 5th). HEL transcript corresponding to probe set (758bp) TaAffx.100436.1.S1_at represents the Gene Index DFCI TC419595 of wheat aestivum and Plant Transcript TA97230_4565.

The results cluster analysis of the expression profiles in Talamè *et al*. (2007) experiment suggest that caution should be exerted when transcriptome information obtained under conditions of water deficit induced in a very short time (e.g. a few hours) is used to understand better how plants may regulate gene expression in response to a water deficit developed under more naturally induced drought conditions; and/or to identify candidate genes for QTLs of field-related traits with an adaptive role in drought. In contrast, a slow decrease in soil moisture would lead to gradual changes in sensing/signalling that, several days after the onset of the drought episode, might return to a pre-stress level. Such drought-stress treatments would then more appropriately measure the result of acclimation reported by transcripts and proteins that provide long-term protection. Experiments that induce water deficit by shock-like dehydration between different cultivars in a comparative fashion have not been conducted. It is thus

unknown how different level of gene expression can be kept depending on the speed of the applied stress.

Ideally, one strategy could be to carry out both types of treatments in time-course experiments for a recording of transcript dynamic changes involved in the response of plants to conditions of water deficit. Shock-like treatments tend to measure the capacity of a plant to respond to a severe and acute change in the environment. This response is based on the regulation of genes involved in the sensing, signalling, and immediate response categories, many of which, while reporting the degree or severity of a stress, might not be translated into bulk changes of proteins. However, the immediate water deficit created by letting the wheat plants wilt is different from the gradual change in shoot water status in intact plants responding to drying soil, and the results document this different behaviour.

3.6 Conclusion

This study was designed to find drought regulated genes in stressed tissues with different behaving (FC) between cultivars Ofanto and Cappelli. For finding genes of interest, results of microarray experiment and literature information were considered. Specific molecular markers were designed to detect these genes. Some drought-induced genes with strong differences between cultivars were found. Secondary objective was comparison of gene expression microarray analysis carried out on stressed tissues in booting stage, with the results of qRT-PCR at the tillering stage. Some genes showing a conserved stress induction profile (in terms of both stress responsiveness and genotype dependence) have been identified during plant development. Genes showing a significant difference of expression level for one cultivar, and also stability across stages of development were selected. These drought induced genes will be used for expression-QTL mapping on RIL population derived from the cross Ofanto x Cappelli.

Chapter Five

EXPRESSION QTL ANALYSIS OF GENES EXPRESSED IN RESPONSE TO DROUGHT IN DURUM WHEAT (*Triticum durum* Desf.) RECOMBINANT INBRED LINES

eQTL Analysis of Genes Expressed in Response to Drought in Durum Wheat RIL Population

4.1. Abstract

Drought is an abiotic stress leading to changes in expression of a large number of plant genes in that strongly influence plant growth, development and productivity. The aim of this research was to characterize the expression variation and the regulatory network of stress-related genes in durum wheat (*Triticum durum*) by quantitative trait loci (QTL) mapping approaches (genetical genomics). In this study we analysed 80 RILs (Recombinant Inbred Lines) derived from the cross Ofanto x Cappelli for the accumulation of two selected drought-related transcripts in order to map expression quantitative trait loci (eQTLs). The association between transcript expression and genotype was tested by using the Ofanto x Cappelli genetic map previously developed at the CRA-Cereal Research Center (Foggia), and a suitable QTL mapping software (MapQTL5). The genomic regions involved in the regulation of stress-related gene expression were identified. These results demonstrate that the genetical genomics approach provides a powerful tool to find association between the genetic bases controlling the molecular response to drought and drought tolerance per se.

Key words: *Triticum durum*, RIL, drought stress, expression analysis, QTL mapping,

4.2 Introduction

Gene expression Quantitative Trait Locus (eQTL) mapping is a technique that measures the association between transcript expression and genotype in order to find genomic locations likely to regulate transcript expression. The availability of both gene expression and high-density genotype data has improved our ability to perform eQTL mapping in Recombinant Inbred Lines (RIL) and other homozygous populations. QTL mapping has been highly useful in identifying causative loci which support several kind

of resistance to biotic/abiotic stress in different crops (Ordas *et al.*, 2010; Ping *et al.*, 2003; Khan *et al.*, 2006; Shen *et al.*, 2006, Langridge *et al.*, 2006, Foster *et al.*, 2004)). Comparative analysis of QTL results clearly shows that chromosomal regions determining variation in agronomic and physiological drought-related traits cover a large proportion of the whole genome. Selection for drought tolerance could become more efficient thanks to the availability of handle markers tightly linked to loci for stress related traits (Cattivelli *et al.*, 2008). Markers diagnostic of individual QTLs represent an important surrogate for physiological trait measurements, and may ultimately improve breeding efficiency through marker assisted selection (MAS).

The recent advances in the molecular investigation of the stress response, particularly after the recent development of the array technology, have led to the identification of several chromosomes (Galiba *et al.*, 1992) or chromosome regions carryng a great number of genes whose expression is associated to drought (Aprile *et al.*, 2008). On the other hand, parallel genetic studies have identified several loci responsible for frost resistance or for component associated to drought resistance. The challenge of the future is to understand the relationship between resistance loci and the expression of stress responsive genes (Cattivelli *et al.*, 2002). We performed an experiment based on the integration of a genetic map, phenotypic and transcript level data to identify genes and loci controlling drought tolerance at tillering stage. In particular, the Ofanto x Cappelli RIL segregating population, derived from two cultivars contrasting for water stress response, was utilized with the corresponding genetic map to identify the expression QTLs.

4.3 Materials and methods

4.3.1 Plant material, RSWC

An experiment was set up with 80 RILs, representing the genetic variability of whole Ofanto x Cappelli segregating population, composed by a total of 161 lines (Figure 16). The seed samples used in this work were kindly provided by CRA-Cereal Research Center (Foggia). Two replications were considered for each RIL. Due to the high number of genotypes utilized, the two parents were replicated 10 times in order to check the stability of gene expression in different points of the growth chamber. Seeds were transferred to square plastic pots with inner dimensions of 7x6x18cm (top diameter,

bottom diameter, and height, respectively) at 0.7 Zadoks stage. The soil was composed of 80% turf and 20% vineyard soil and fertilizer was added. Each pot was filled with 400 g of soil, then watered to excess, covered with a thin transparent plastic film and allowed to drain until reaching a constant weight, which was determinate as water holding capacity (the saturated weight, W_S) The pots contained drainage holes, but were supplemented with aluminum covers to prevent any water leakage. In addition, 400 g of soil from four extra pots was placed in a forced air drier at 150 °C for 24 hours, to estimate the oven-dry weight of soil per pot (typically 221-217 g). Sum of the soil dry weight, the pot weight and the lids weight was used as the "dry weight" value (W_D). Relative soil water content (RSWC) was calculated as:

$$RSWC = ((\text{current pot weight} - W_D) / (W_S - W_D)) * 100$$

Seedlings were transplanted one to a pot and were grown in a growth chamber Sanyo, where temperatures were maintained at 20 °C during the day and 15 °C during the night. Photoperiod was 12 h (6 h – 18 h day, 18 h – 6 h night) with average quantum flux density of PAR of 400 µmol quanta m^{-2} sec^{-1} during a 12 h day and humidity of 70% -90% during the day and night respectively. Water was withheld from wheat plants during 32 day of experiment (DOE) until approximately tillering DC 21-25, to achieve a severe water stress at 27/29% RSWC level in all plants, both parentals and RILs.

4.3.2 RNA extraction and cDNA synthesis

Total RNA was isolated from uppermost fully expanded leaves (5th leaf) after achieving 27/28% RSWC using the TRI Reagent® Solution (Ambion; for details see Appendix 3). Three hundred mg of leaf tissue were used for each sample. RNA concentration[1] and purity[2] were checked with a Beckman DU®640 spectrophotometer before and after RNA treatment[3]. The RNA concentration was determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA). Only RNA samples

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[1] To determine the RNA concentration in µg/ml, was the A_{260} by the dilution factor and the extinction coefficient multiplied (1 A_{260} = 40 µg RNA/ml). **A_{260} × dilution factor × 40 = µg RNA/ml**

[2] The A_{260}/A_{280} ratio of the RNA is an indication of its purity. The total RNA isolated with this procedure should have an A_{260}/A_{280} ratio of 1.8-2.2.

[3] RNA concentration was measured after RNA extraction and then after RNA Cleanup and DNA-free treatment, to determine the residual contamination.

with 260/280 wavelength ratio between 1.9 and 2.1 were used for cDNA synthesis. For total RNA cleanup the Qiagen RNA Cleanup Protocol was used (see Appendix 4) while the Ambion's DNA-*free*™ Kit was used for removing the contaminating DNA, the DNase and divalent cations from the sample (Appendix 5). After purification, all samples were tested for the presence of residual DNA by using 20 ng of the RNA as a template in a PCR reaction with the primer pairs utilized in the experiment. Finally, the integrity and suitability of RNA for cDNA synthesis was assessed by loading 40 representative samples on the Agilent® RNA Nano 6000 chip and resolving them on an Agilent® 2100 BioAnalyzer according to the manufacturer's instructions (for details see Appendix 6). The cDNA was synthesized with Standard Reverse Transcription Protocol (Promega, Appendix 7). For quantification of cDNA samples, Quibit fluorometerTM (Invitrogen) was used (Appendix 8).

4.3.3 qRT-PCR analysis

Quantitative real time polymerase chain reaction was performed with SYBR Green fluorescence detection in a qPCR thermal cycler (ABI PRISM 7300, Applied Biosystems). Each reaction was prepared using 3 µl from a 2 ng/µl dilution of cDNA derived from the RT reaction, 12.5 µl of SYBR Green PCR 2x Master Mix (Qiagen), 1 µM forward and reverse primers, in a total volume of 25µl. For each primer pairs two controls with no template were run to detect contamination and dimer formation. The cycling conditions contained: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, with the final dissociation at 95 °C for 15 sec, 60 °C for 30 sec and 95 °C for 15 sec. Melting curve analysis was performed to evaluate the presence of non-specific PCR products and primer dimers. Two wheat genes from PLEXdb[1] databases: *cyclophilin* (Accession number Ta.27454.2.S1_a_at) and mitochondrial import inner membrane translocase subunit *TIM17* (Accession number Ta.12727.1.S1_at) were used as internal reference genes for relative expression quantification. Three technical replicates for each sample were used.

To confirm the differential expression levels between cv. Ofanto and Cappelli for two candidate genes listed in Table 9, a qRT-PCR analysis was first performed only on parental samples. The different expression level in the two parents was confirmed for

[1] http://www.plexdb.org/

both genes (*HEL* and *TaFBPase_1*), therefore the evaluation was extended to the 80 RILs in order to obtain genotypic data to map the correspective eQTLs. The amplification products corresponding to both genes were analyzed with gel electrophoresis and sequenced by using a AB3130 sequencer in order to prove their identity (see Appendix 10).

Table 9 Sequences of the specific primers for candidate and reference genes and source of sequence/publication.

Primer	Nucleotide sequence forward	Nucleotide sequence reverse	Lenght (bp)	Source
Reference genes				
TIM17	5'-GATGCACCTAGTTCCTTTGCAA-3'	5'-GCAAGATATTTATTCCAGGTCTGTTG-3'	81	Ta.12727.1.S1_at
Cyclophilin	5'-CCGATGAGAACTTCAAGCTCAA-3'	5'-GCATTGGCCATGGAAAGGT-3'	58	Ta.27454.2.S1_a_at
Target genes				
TaFBPase_1	5'-ACACCGTGACTCTGGAGGAAG-3'	5'-AAGAGAAGGGTCAAGCGTGAAG-3'	139	Xue *et al.*, 2008
HEL	5'-GGATTGTCAGGATGGCTATGTG-3'	5'-TCAGTAAGACCAACACCATTTTTAGG-3'	96	TaAffx.100436.1.S1_at

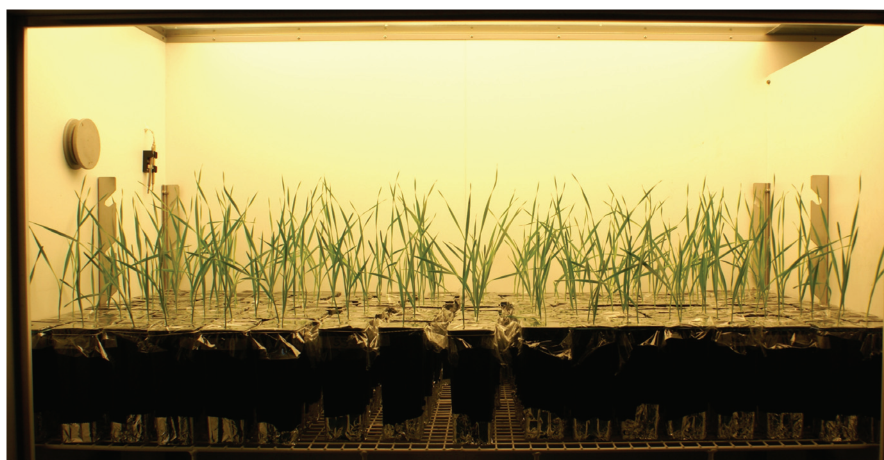

Figure 16 RILs population derived from the cross Ofanto x Cappelli in the grown chamber.

4.4 Results

Stability and level of gene expression of two drought-induced candidate genes was first confirmed in parental samples by qRT-PCR analysis. The data confirmed that the *HEL* gene sequence was much more induced in Ofanto than in Cappelli in severe drought stress, while *TaFBPase_1* was expressed at higher level in Cappelli than in Ofanto (Figure 17). Sequences of these genes are listed inTable 10.

The transcripts accumulation for the two genes was then determined in the 80 RILs subjected to drought stress in order to identify genomic regions controlling their expression. The transcripts amount was expressed as ΔCt values ($\Delta C_t = C_{t\ target\ gene} - C_{t\ endogenous\ gene}$).

Table 10 Sequences of *HEL* and *TaFBPase_1* genes obtained from sequencing analyses, grey boxes indicates qRT-PCR primers and primers used for sequencing are underlined.

Gene	Nucleotide sequence
HEL	GCAACGGCAGAGGTCATTATACTGACCAAGTCAGGGAGTCAAAGGTGATGGATTGTCAGGATGGCTATGTGGAGAGATGC ATACGCATCGGTATTACCGGCATTATCTGCCGTTGCTTTCCTAAAAATGGTGTTGGTCTTACTGACCAAGCAATGTTGGCG ACGGCTGCCTCACCGAGTAAGATTGACGAGGAGAGACCGTCACCGAAAAACTCCCACGACCA
TaFBPase_1	TACACCGTGACTCTGGAGGAAGTACTGCAGCCTGGGAAGGACATGACTGCTGCCGGATACTGCATGTATGGGAGTTCCTGC ACGCTTGTCCTGAGCACTGGAAATGGTGTCAACGGCTTCACGCTTGACCCTTCTCTT

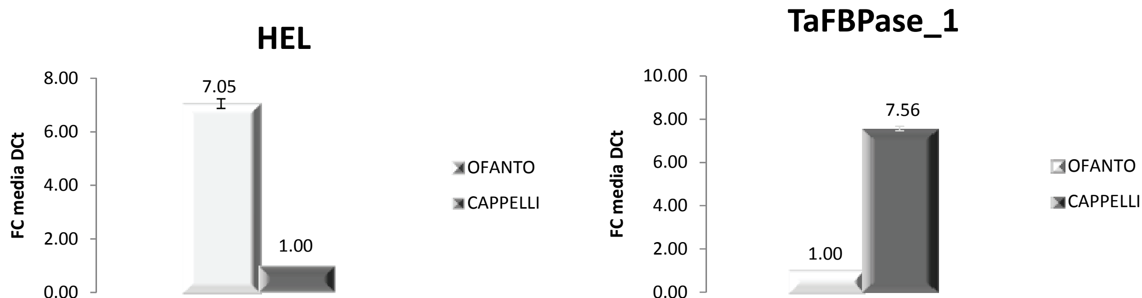

Figure 17 qRT-PCR analysis in parental samples, where *HEL* gene sequence was much more induced in Ofanto than in Cappelli in severe drought stress (27/28% RSWC), while *TaFBPase_1* was expressed at higher level in Cappelli than in Ofanto.

Regarding the *TaFBPase_1* gene, all tested lines showed negative values of ΔCt as found in the parent Cappelli, indicating a strong distortion in the segregation of this trait, and no QTL was identified following the MQM analysis.

The expression level of the *HEL* gene showed a good segregation among lines, with a frequency distribution indicating a simple genetic control of the trait (Figure 18).

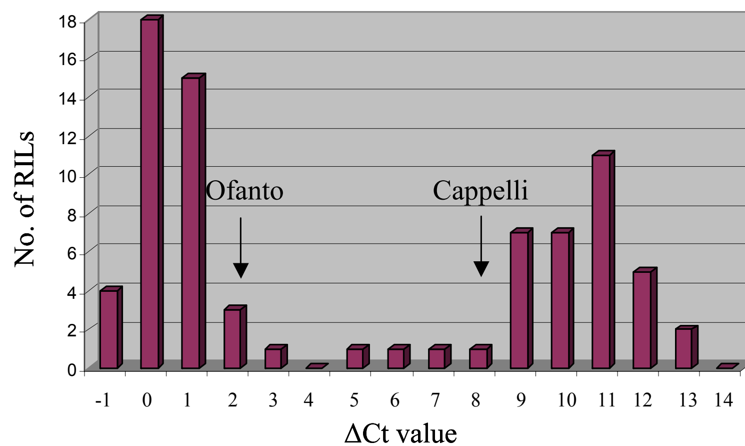

Figure 18 Frequency distribution of ΔCt values in the 80 RILs analyzed.

The QTL analysis carried out for the *HEL* gene showed that the expression level of this gene was controlled by a major locus localized on the telomeric region of the long arm of chromosome 6B (Figure 19).

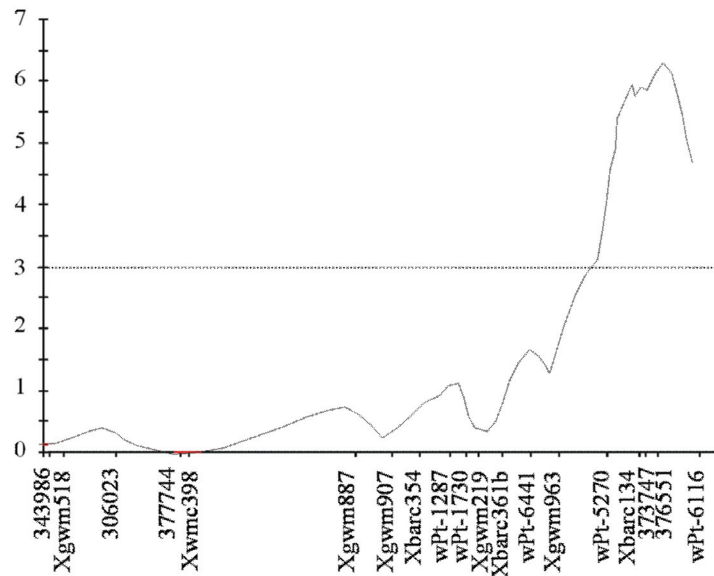

Figure 19 Genetic map of the region on chromosome 6B carrying the major QTL controlling the expression level of the *HEL* gene, and the corresponding curve of the LOD values.

The eQTL identified in this study showed a LOD value of 6.3 in correspondence of the DArT marker 376561, and explained about 36% of the observed variability for the trait. A negative additive effect was found, which indicated that the allele carried by Ofanto was effective in decrease of ΔCt value of 3.04 units, and therefore in increasing the *HEL* gene expression level.

Sequence alignment analysis of *HEL* gene was done by using sequences which have been obtained from "blastn" submission in cerealsDB[1] databases showed similarity with Chinense spring contig 444272 (Length = 1448, Score = 361 bits (182), Expect = 5e-97, Identities = 198/203 (97%), Gaps = 1/203 (0%), with TIGR Plant Transcript Assembly[2] (Plant Transcript TA97230_4565) and DFCI Wheat Gene Index database[3] (Tentative Consensus TC419595) were used. Complete sequence from Plant Expression Database – PLEXdb[4] was also added. All blast results did not provide any information about gene annotation. Not even research for ortholog genome sequences in close related species as *Brachypodium*[5] and *Oryza*[5], for coding region detection in DNA sequences using ESTScan[6] or searching for protein sequence in UniProt[7] was

[1] http://www.cerealsdb.uk.net/index.htm
[2] http://plantta.jcvi.org/index.shtml
[3] http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi
[4] http://www.plexdb.org/index.php
[5] www.gramene.org, www.phytozome.net
[6] http://www.ch.embnet.org/software/ESTScan2.html
[7] www.uniprot.org

successfully performed. Otherwise, alignment of these sequences detected putative intron (Figure 20). This fact open the possibility of using it as candidate target to find polymorfisms between two parental Ofanto and Cappelli which did not show differences in their cDNA sequences.

Figure 20 Alignment analysis of contig 444272 sequence (1500bp) with the homologous sequences TA97230_4565, TC419595 and TaAffx.100436.1.S1_at. From the alignment, carried out using the AlignX programm from the suite VectorNTI (Invitrogen), results that the contig444272 sequence contains an intron in the region from 372 to 493 bp.

4.5 Discussion

Many studies are available in literature about linkage mapping of genomic regions involved in the control of traits agronomically important, but very few are still the evidences about eQTLs, that is QTLs explaining the accumulation of the transcript of interest. In this study we identified an eQTL explaining a great proportion of the observed variability for the expression of the *HEL* gene in a subset of the durum wheat segregating population Ofanto x Cappelli. Despite it was not possible to carry out the experiment on the whole population composed by 161 RILs, the eQTL was statistically significant and robust with a LOD value of about 7.

The *HEL* gene is regulated by drought and the resistance to a mild water stress is largely recognized as a trait sustaining the grain yield in field conditions, a factor very important to fill in the gap between potential and actual yield in mild water stressed environments (Fleury *et al*., 2010). Interestingly, some QTLs for traits related to grain yield were previously identified in the region of the eQTL found in this study, based on molecular markers in common with published genetic maps.

In particular, Neumann *et al*. (2010) found a statistically significant association between a DArT marker positioned in the telomeric region of chromosome 6BL and grain weight per spike. In another association mapping study, Maccaferri *et al*. (2011) identified a significant association between the *Xbarc134* marker, (very near to the peak marker in our genetic map) and the number of grains per square meter. Based on these results, the *HEL* gene could be involved in biological mechanisms controlling grain yield related traits in conditions of low water availability.
Further studies are in course in order to clarify if the identified chromosomal region contains the *HEL* gene or a regulator of its expression.

4.6 Conclusion

Many studies have focused on the study of understanding plant responses to drought and an increase in drought tolerance was achieved by breeders specially by using empirical selection. This practice was followed with an idea of linking physiology and quantitative genetics. Mapping of a drought induced genes responsive for expression pattern or genes that may encode an enzyme or protein involved in a metabolic pathway can have a strong impact for further improving of integrative

approach. We enhanced our knowledge of the mechanism of drought tolerance by research program targeting on study of transcript of interest and their accumulation as eQTLs. This approach can together with sequencing, marker development and genome-wide analysis more likely control the way in which we structure populations for analysis and for solving specific components of drought tolerance. Phenotyping is becoming to be most expensive, time consuming and most limiting step in the genetic analysis of drought tolerance and other traits. Development of rapid and cheap procedures to characterize components of the drought response is crucial for genetic improvement.

Chapter Six

THE DEVELOPMENT OF SPECIFIC MOLECULAR MARKER FOR DETECTION OF SELF-INCOMPATIBLE PLANT – A POWERFUL TOOL IN *Brassica napus* L. HYBRID BREEDING

The Development of Specific Molecular Marker for Detection of Self-Incompatible Plants – a Powerful Tool in *Brassica napus* Hybrid Breeding

5.1 Abstract

Self-incompatibility (SI) is a mechanism which prevents self-fertilization by inhibiting pollen tube growth of self-pollen either on the stigma surface or in the style-transmitting tissue. Self-incompatibility in the *Brassicaceae* family is sporophytically controlled by a single multi-allelic locus (*S* locus), which contains at least three highly polymorphic genes expressed in the stigma (*SLG* and *SRK* genes) and in the pollen (*SCR*/*SP11* gene). SI could be used as a pollination control system for *Brassica napus* F_1 hybrid breeding, if a sufficient number of *S* alleles is available in this species. Although the diploid species *Brassica rapa* (syn. *campestris*; donor of A genome) and *Brassica oleracea* (donor of C genome) are typically self-incompatible, *B. napus,* an allotetraploid composed of both of these genomes, generally occurs as a self-compatible (SC) plant.

Czech breeder developed a unique set of SI doublehaploid (DH) lines with stable recessive S alleles derived from crosses between SI donor lines and self-compatible donors of 00-quality. In this study we have used three *S* genes, class I *SLG* gene, class II *SCR* gene and *SRK* gene, as molecular markers in order to screen segregating F_2 population for SI plants. We also tested the stringency of these molecular markers and co-segregation of molecular markers and SI/SC trait. The specific molecular marker on the basis of newly determined class II *SCR* gene allele (SCRII-Tandem 2CB allele) was developed. This marker specifically determines SI plants derived from line "SI-Tandem" only. In contrast to *SCR* II marker gene, universal *SLG* I and *SRK* marker genes enables to detect SC plants in segregating populations. Theoretically expected segregation ratio 1:1 (SI:SC) was confirmed by molecular analysis in two model populations.

Key words: *Brassica napus*, self-incompatibility, MAS, *S* locus

5.2 Introduction

In the *Brassica* self-incompatibility system, the self-recognition specificity between the pollen and the stigma is controlled by a single polymorphic locus, the *S* locus (Bateman, 1955). There are at least three highly polymorphic genes at the locus: *SLG* (*S* locus glycoprotein gene) (Nasrallah *et al*., 1988), *SRK* (*S* locus receptor kinase gene) (Stein *et al*., 1991) and *SCR*/*SP11* (*S* locus cysteine rich protein gene) (Schopfer *et al*., 1999; Suzuki *et al*., 1999). *SLG* gene encodes a highly polymorphic secretory glycoprotein that is localized in the cell wall of stigma papillar cells (Nasrallah *et al*., 1985). *SLG* has a sequence similar to the extracellular domain (*S* domain) of *SRK* (Stein *et al*., 1991). SRK is the sole determinant of specifity in stigma, and encodes a membrane-associated receptor protein kinase with extracellular, transmembrane and cytoplasmic kinase domains (Takasaki *et al*., 2000; Silva *et al*., 2001). SCR/SP11 is the male determinant of *S* locus specifity in the polen coat and encodes a low-molecular weight cysteine-rich protein specifically expressed in the anther tissues (Shiba *et al*., 2001). These three genes are transmitted to progeny as a single transcription unit, and therefore "*S* allele" is refered to as "*S* haplotype" (Nasrallah and Nasrallah, 1993). *S* haplotypes were classified into two classes with respect to the nucleotide sequence similarity of *SLG* and *SRK* alleles. Class I *S* haplotypes are known to be generally dominant to class II *S* haplotypes in pollen (Nasrallah *et al*., 1991). Although the diploid species from "*Brassica* triangle" (Labana and Gupta, 1993; Morinaga, 1934; U, 1935) are typically self-incompatible, *B. napus,* an allotetraploid composed of the A and C *Brassica* genomes, generally occurs as a self-compatible plant (Downey and Rakow, 1987). SI plants could be found in old population cultivars (Havel, 1996) as well as in modern cultivars and breeding materials as a result of extensive molecular screening (Čurn *et al*., 2008). Plants with functional *S* alleles have been included into Brassica hybrid breeding programmes and molecular techniques play the crucial role not only as a tool for discovering new alleles, or gene resources with new alleles, but also as a tool for developing selectable markers which allows to determine SI plants in early ontogenetic stages.

5.3 Materials and methods

5.3.1 Plant material

Seed of the cultivars was obtained directly from the breeding stations Opava and Slapy, Czech Republic. DH lines were regenerated via a microspore embryogenesis procedure from the SI plants with objective to fix SI phenotype and low glucosinolate content at the Research Institute of Crop Production in Prague, Czech Republic.

The segregating population of DH lines was derived from three crosses between SI line AIK 6 and self-compatible (SC) cultivar Rasmus, SI line AIK 3 and SC line OP-571/00 and finally SI line AIK 6 and SC line OP BN-03. AIK 3 and AIK 6 are DH lines originated from crosses between a donor of recessive self-incompatibility (Tandem) and donor of 00-quality (OP-1051) (chart of the origin of DH lines see on Figure 21). Segregating population of 120 plants was analyzed by PCR using class I *SLG*-specific primers and 76 subsequently selected plants showing SI genotype according to molecular analyses were also checked for their phenotypic expression (number of seeds per silique so-called "seed test" (Vyvadilová *et al.*, 2008, Kučera *et al.*, 1999). Plants resp. branches were covered by bags 3 days before flowering and average numbers of seeds per developed silique after self-pollination in flowers were counted. The seed set by selfing was checked when silique were ripen. Plants were considered as SI when average number of seeds per silique was ranging from 1 to 10, as partially SI with average from 10 to 30 and completely SC with more than 30 per silique.

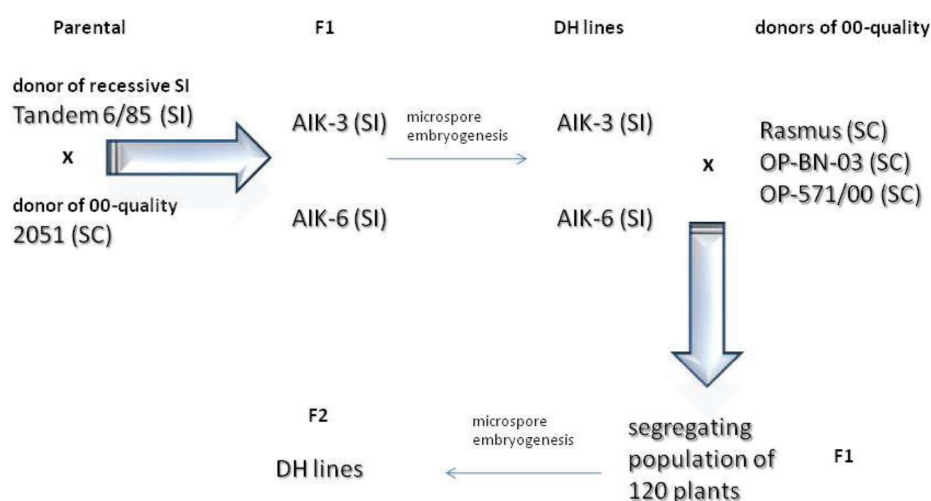

Figure 21 Production of *Brassica napus* dihaploid lines.

5.3.2 DNA extraction, PCR amplification

Genomic DNA of *B. napus* cultivars and DH lines was extracted from young leaves of 2-week-old seedlings using the DNeasy Plant Mini kit (Qiagen). Amplification of specific fragments of *SLG, SCR* and *SRK* genes was following: PCR was performed with 2 µl (40 ng) of genomic DNA as template, mixed with 0,25 µl (0,5 µM) of each primer (see Table 11), 12,5 µl SYBR MM (qPCR 2x SYBR Master Mix, TopBio) and distilled water to make a final volume of 25 µl.

PCR reaction with class I ***SLG***-specific primers PS5 and PS15 (Nishio *et al*., 1996) involved pre-denaturaction for 5 min at 94°C, 35 cycles of 1 min at 93°C, 2 min at 58°C and 3 min at 72°C, finally, 10 min at 72°C.

SCR gene was amplified with class II *SCR*-specific oligonucleotide primers 2a and 2b designed for functional allele originating from SI line Tandem, SCRII-Tandem 2CB allele (Žaludová, 2007). PCR involved 35 cycles of 30 s at 94°C, 30 s 60°C and 1 min at 72°C.

SRK gene was amplified with class I *SRK*-specific primers; forward primer PK1 having the nucleotide sequence of the second exon of S^6 *SRK* and a reverse primer PK4 having the nucleotide sequence complementary to the fifth exon of S^6 *SRK* (Nishio *et al*., 1997). PCR involved 30 cycles of 1 min at 94°C, 2 min 55°C and 3 min at 72°C. For amplification of *SRK* alleles of the class II *S* haplotypes, specific primers PK7-2II and PK8-2II (Fukai *et al*., 2003) were used. PCR condition involved pre-denaturaction for 2 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C, finally, 10 min at 72°C.

All of PCR products were subjected to electrophoresis on 1,5% agarose gel in 1 × TB buffer, and detected by staining with ethidium bromide.

Table 11 Sequences of the *S* locus specific primers.

Primer	Nucleotide sequence	Source
SLG		
PS5	5'-ATGAAAGGCGTAAGAAAAACCTA-3'	Nishio *et al*., 1996
PS15	5'-CCGTGTTTTATTTTAAGAGAAAGAGCT-3'	Nishio *et al*., 1996
SCR		
2a	5'-TTGGACTTTGACATATGTTC-3'	Žaludová, 2007
2b	5'-CTCTGAAGTGGGTTTTACAG-3'	Žaludová, 2007
SRK		
PK1	5'-CTGCTGATCATGTTCTGCCTCTGG-3'	Nishio *et al*., 1997
PK4	5'-CAATCCCAAAATCCGAGATCT-3'	Nishio *et al*., 1997
PK7-2II	5'-ATGAAAAGGGTACAGAACATTTACCACC-3'	Fukai *et al*., 2003
PK8-2II	5'-CCAGTTCGGTCTCTCTTCTCACCCGAGG-3'	Fukai *et al*., 2003

5.3.3 PCR - RFLP

Electrophoretic analysis of cleaved DNA fragments:

SRK gene was also analyzed by polymerase chain reaction restriction fragment length polymorphism method (PCR-RFLP). PCR was performed using PK7-2II and PK8-2II primers, and amplified products were digested with restriction endonucleases *Eco*RI, *Mse*I and *Mnl*I at 37°C for 2 h. Restriction digestion conditions were as follows: 10 µl PCR product, 2 µl of 10 × buffer and 1 unit of restriction enzyme in a final volume of 15 µl. The products were separated by electrophoresis on 2,5% agarose gel in 1 × TBE buffer at 90 V for 2,5 h and, DNA bands were visualised by ethidium bromide staining.

5.4 Results and discussion

Three genes were used as molecular marker for selection of self-incompatible plants. For all genes numbers of different specific primers of the class I and class II were tested and among them, successful primers for each gene were selected as was described above. PCR with class I *SLG*-specific primers has resulted in approximately 1300 bp fragment (Figure 22). This fragment was specifically present in plants considered to be self-compatible. This molecular marker was detected in a wide range of naturally self-compatible rapeseed cultivars whereas it was not present, neither in self-incompatible DH lines, nor in SI donors. As the analysed rapeseed lines are characterised by recessive type of self-incompatibility, the dominant class I *SLG* gene was not amplified in putative SI plants because of a dysfunction conferred to self-compatible rapeseed plants. Because it was proved that *SLG* gene is not essential in pollen-stigmas recognition reaction (Okazaki *et al*., 1999; Suzuki *et al*., 2000) and considering that SLG protein is not essential in pollen-stigma recognition reaction (Nishio and Kusaba, 2000), the absence of class I *SLG* gene could suggest the lack of all class I *S* locus which could be somehow affected (Suzuki *et al*., 2000).

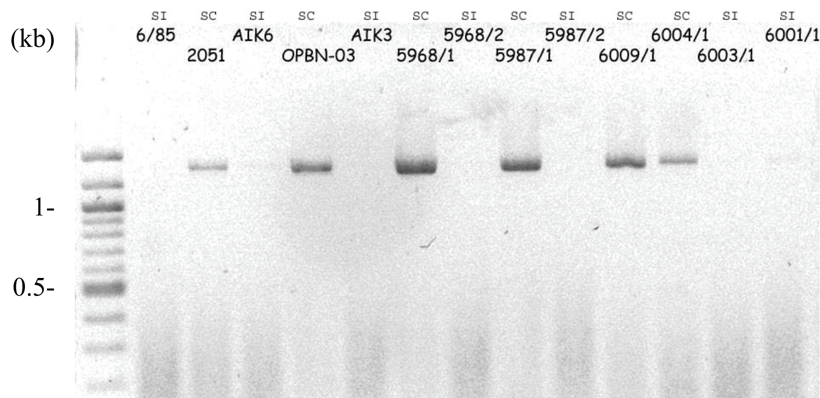

Figure 22 Amplification of class I *SLG* gene. PCR with class I *SLG*-specific primers (PS5 and PS15) has resulted in approximately 1300 bp fragment which are specifically present in plants considered to be self-compatible.

The results and also the character of *SLG* marker gene (unclear function in self-incompatibility reaction) indicate that additional marker system would be necessary, to enable molecular based selection of desired plants. This second approach was to use a functional allele of essential gene in SI reaction and this marker system was specifically targeted on alleles of class II *SCR* gene. SCR is sole pollen determinant of self-incompatibility (Schopfer *et al*., 1999; Suzuki *et al*., 1999). It has been confirmed that *SCR* gene is duplicated in *Brassica oleracea* S^{15} haplotype (Shiba *et al.*, 2004). Cabrillac *et al*. (1999) has found duplicated *SLG* gene in SC *Brassica oleracea*, line S^{15}. We found two alleles in our analysed population: the first duplicated allele of *SCR*, which was 100% similar to S^{15} alele from *Brassica oleracea*. This could suggest that this allele originates from *B. oleracea*. The duplication could lead to the producing of incorrect lengths of transcripts responsible for dysfunction (Shiba *et al.*, 2004). The second allele was specifically occurred in SI lines ("allele 2", Žaludová 2007). This approach was supposed to be more accurate than application of *SLG* marker gene. After comparison of the first marker system (*SLG* marker gene) with the second (*SCR* marker functional gene) perfect match was obtained between these two markers (markers corresponded each other) on the level of parental plants, F_1 population and DH lines representing segregating population after first cycle of crossing. After second cycle of crossing (i.e. in segregating population derived from crossing of AIK-3/AIK-6 with Rasmus/OP-BN-03/OP-571/00), was not detection of *SCR* gene successful probably due to the presence of gene modifiers or more complex genetic background derived from SC donors of 00-quality. However, we could not exclude

the possibility that there are another oilseed rape plant with functional class II *SCR* gene and dysfunctional class I *SLG* gene simultaneously. In that event, class I *S* locus containing *SLG* gene, considered as a dominant, should suppress functional class II *S* locus with *SCR* gene resulting in common self-compatible oilseed rape plant. Due to the influence of possible negative background from SC donors of 00-quality, it seems to be useful eliminate donors which contains non-functional alleles and can provide false signal during screening of population after second cycle of crossing. Further attention was therefore focused on utilization of third gene of *S* locus (*SRK*) with effort to find a suitable molecular marker.

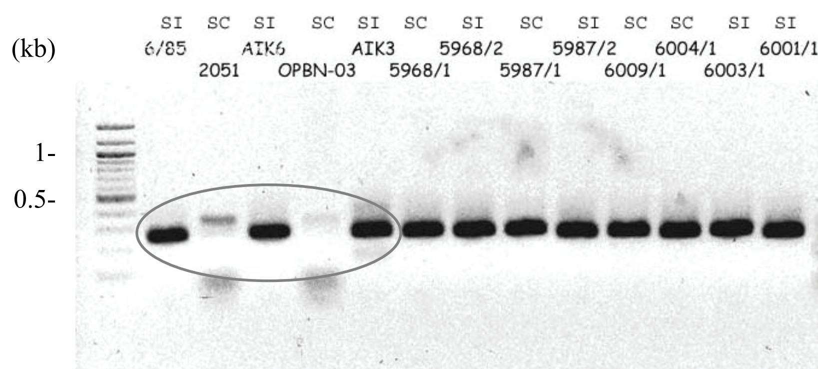

Figure 23 Amplification of class II *SCR* gene. An 280 bp amplicon detected putative self-incompatible oilseed rape plants only on the level of parents and DH lines after first cycle of their crossing (AIK6 and AIK3) (in gray circle). After second cycle of crossing was not possible recognize SI plants on molecular level.

The SCR allelic specific marker was developed after analyses of self-incompatible line derived from Tandem. The amplified fragment coresponding to recesive allele of *SCR* II gene was 280 bp long and specifically occurred in plants considered to be self-incompatible (Figure 23). The marker demonstrated the successful selection of SI plants only in “Tandem descent”. In the applied breeding programme, Tandem derived SI lines (AIK3 and AIK6) are further improved via several cycles of crossing with elite 00-quality donors. SC 00-quality donors probably hold gene “modifier” and regular segregation rate of SC/SI plants is modified and the allele specific molecular marker can be used especially for selection of SI donor plants and DH lines after the first cycle of crossing (Žaludová, 2007).

Primer pair PK1 and PK4 for *SRK* gene was found to be the best combination for the amplification of polymorphic DNA fragments of *SRK* alleles. PCR reaction using this primer pair gave a single DNA fragment ranging from 0.9

to 1.0 kb. This fragments detected all self-compatible plants (as in the case of class I *SLG*-specific primers) (**Chyba! Nenalezen zdroj odkazů.**). PCR products amplified with PK7-2II and PK8-2II were fragments of about 2 kb generated in all tested plants, but CAPS analysis resulted in successful differentiation of SI/SC plants on the level of donors 00-quality and SC DH lines with all the restriction endonucleases (*Eco*RI, *Mse*I and *Mnl*I) (data not shown). As in the case of *SCR* II gene, this system can be used for efficient selection of parental plants and plants after the first cycle of improve breeding (SI donor lines x donor quality).

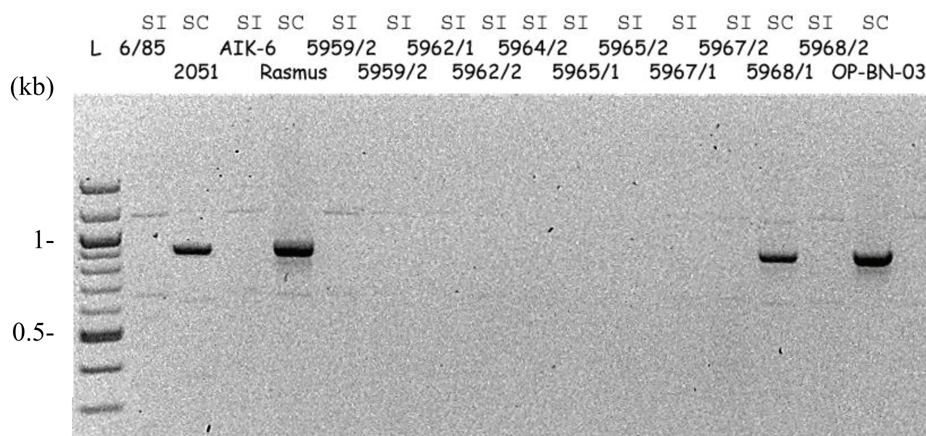

Figure 24 Amplification of *SRK* gene by using PK1 and PK4 primers. DNA fragments from 0.9 to 1.0 kb detected self-compatible plants.

5.5 Conclusion

Class I *SLG* molecular marker gene tagged 80% SC plants excluding SI/SC phenotype allowing rapid pre-screening appropriate to exclude unwanted SC plants, without loss of genetic material in the form of SI plants. However this selection system detecting SC plants can be supplemented by using of additional molecular marker of *SRK* gene providing control of efficiency of selection. Marker system detecting SC plants in this study appears to be more useful for large scale screening of breeding materials than specially developed *SCR* marker originating from particular SI line. In the case of *SCR* and *SRK* genes, detected with 2a, 2b and PK7-2II, PK8-2II primers respectively, phenotype segregating ratio did not correspond with molecular marker determined genotype in second cycle of crossing, probably due to some modifications. On the basis of phenotype segregation ratio two different loci coding SI should exist. One of them is the *S* locus with two classes of alleles. Class I *S* locus was present only in SC plant and

moreover in all SC cultivars (data not shown). It could suggest that class I *S* locus is in some way affected and in plants is replaced with functional class II *S* locus. The second locus is repressor locus (R-locus), which is commonly present in SC oilseed rape cultivars and not present in SI lines. Ekuere *et al.* (2004) describes a suppressor loci with suppressing alleles in oilseed rape and non-suppressing alleles in diploid parents of resynthesized *B. napus* lines that are not linked to the *S* loci. Both of these loci could independently affect SI. As a lot of intermediate SI/SC phenotypes were observed, we should suggest that both *S* locus and R locus have alleles with different recessive or dominant interaction. Development of specific *S* haplotype marker could be useful as a prevention of contamination with SI line carrying different *S* allele and also for precise selection it will be necessary to develop allelic specific markers for each donor of SI.

It can be concluded, that molecular markers based on analysis of *SLG* I, *SCR* II and *SRK* I and II genes can be successfully used for selection of SI plants and undesirable SC plants are eliminated at early ontogenetic stage. Additionally comparative analysis of all of these molecular markers for individual genes can achieve successful selection of functional carriers of recessive *S* alleles during breeding programmes.

Chapter Seven

CONCLUSION

Conclusion

The presented study has showed utilization of different DNA markers to provide essential insights into how genes behave in different environments and in different genetic backgrounds and how to increase efficiency and reliability of selection. The details of fulfilment of the objectives that were mentioned at the beginning of my work follow.

1. Identifying drought-induced genes in two durum wheat (*Triticum durum*) cultivars with contrasting response to drought stress.

1.1. Transcriptomic analysis based on an Affymetrix array platform of plants exposed to drought, heat and to combination of heat and drought.

From a QT-cluster analysis 7 clusters have identified 1850 stress responsive genes characterized by significantly different stress expression profiles in Cappelli vs. Ofanto.

1.2. Data analysis and identification of genes induced or more induced in one genotype compared with the other under the influence of drought stress.

Seventeen drought response gene markers were selected according to the fold change threshold of the Affymetrix dataset and the annotated activity among these genes. In addition another six promising genes were chosen from literature for testing by qRT-PCR experiment.

1.3. Primer designing to detect candidate drought-induced genes and validate their expression profile in new experiment by qRT-PCR.

From the origin 22 genes of interest based on the results of the PCR and qRT-PCR standard validation experiment 15 couple of primers from microarray plus other 5 primers for other drought-related candidates gene detection were chosen to test

their expression level on Ofanto and Cappelli by qRT-PCR analysis. Two wheat genes: *cyclophilin* and *TIM17* were used as internal reference genes for calculation of relative transcript levels of the genes under study. Of the 20 drought-induced genes, 9 (45%) were greater than two-fold up-regulated under water stress in Ofanto and 2 (10%) in Cappelli; the remaining were not significantly (two-fold) up- or down-regulated between two cultivars under drought-stress. Overall, after comparison of gene expression as a result of stress in both cultivars, where are 12 of 20 genes expressed, Ofanto showed much higher genes expression, than Cappelli and this also corresponded with results of previous Affymetrix experiment.

2. Expression QTL analysis of candidate drought-induced genes in durum wheat (*Triticum durum*) recombinant inbred lines (RILs).

2.1. Expression analysis of candidate genes carried out on RILs population to obtain quantitative data describing the expression profiles in response to stress.

Two candidate genes (*HEL* and *TaFBPase_1*) showing stabile level of expression and large difference in the expression profile in response to stress in the two cultivars, have been tested for an expression analysis carried out using a population of 80 RILs in order to obtain genotypic data to map correspective eQTLs. Regarding the *TaFBPase_1* gene, all tested lines showed negative values of ΔCt as found in the parent Cappelli, indicating a strong distortion in the segregation of this trait. The expression level of the *HEL* gene showed a good segregation among lines, with a frequency distribution indicating a simple genetic control of the trait.

2.2. Using this data to map QTL controlling the expression of the drought induced genes.

The QTL analysis carried out for the *HEL* gene showed that the expression level of this gene was controlled by a major locus localized on the telomeric region of the long arm of chromosome 6B. For *TaFBPase_1* gene, no QTL was identified.

2.3. Identifying of eQTL controlling the expression of stress induced genes, co-segregating between eQTLs and QTLs for drought tolerance evaluated on the bases of field trials.

A specific software for QTL mapping of molecular marker map deriving from the cross Ofanto x Cappelli (already available) was used to identify eQTL. The eQTL identified for the *HEL* gene showed a LOD value of 6.3 in correspondence of the DArT marker 376561, and explained about 36% of the observed variability for the trait. A negative additive effect was found, which indicated that the allele carried by Ofanto was effective in decrease of ΔCt value of 3.04 units, and therefore in increasing the *HEL* gene expression level. All blast results did not provide any high identity value for annotation of this gene. Otherwise, alignment of blast sequences detected putative intron. This fact open the possibility of using it as candidate target to find polymorfisms between two parental Ofanto and Cappelli which did not show differences in their cDNA sequences.

3. Aplication of molecular markers for detection of self-incompatibility in oilseed rape (*Brassica napus*).

3.1. Screening of segregating F_2 population for SI plants by using available gene markers for *SLG*, *SCR* and *SRK* genes.

For each of genes *SLG*, *SCR* and *SRK* some set of specific primers was tested. Among them was the PCR reaction optimized for 4 reliable couple of primers (covering all genes) which have been used for screening of tested segregating F_2 population.

3.2. Validation of the molecular markers with phenotypic expression of the plants.

Two molecular markers for *SLG* and *SRK* gene were detected in a wide range of naturally self-compatible rapeseed cultivars whereas were not present in self-incompatible DH lines, nor in SI donors. The first molecular marker for *SLG* gene detection of SC tagged 80% plants compared with the expression of their phenotype. Subsequently second molecular marker for *SRK* gene detected all pre screened SC plants. Marker for *SCR* gene detected self-incompatible plants on the level of SI donor plants and DH lines after first cycle of crossing, but SI detection in

segregating population was probably modified by the presence of gene modifiers derived from SC 00-quality donors.

3.3. Comparison analysis between the markers with the aim to improve combination ability between them to refine selection of SI plants.

Two marker genes *SLG* and *SRK*, which were used for SC detection, correlated each other. Additional molecular marker for detecting SI plants corresponded to the level of SI donor plants and DH lines as was mentioned above and also this marker may also be useful for genotyping of SI donor plants and DH lines after first cycle of crossing.

Chapter Eight

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Chapter Nine

APPENDICES

Appendix 1

The evolution of the *Triticeae* species from a common ancestor

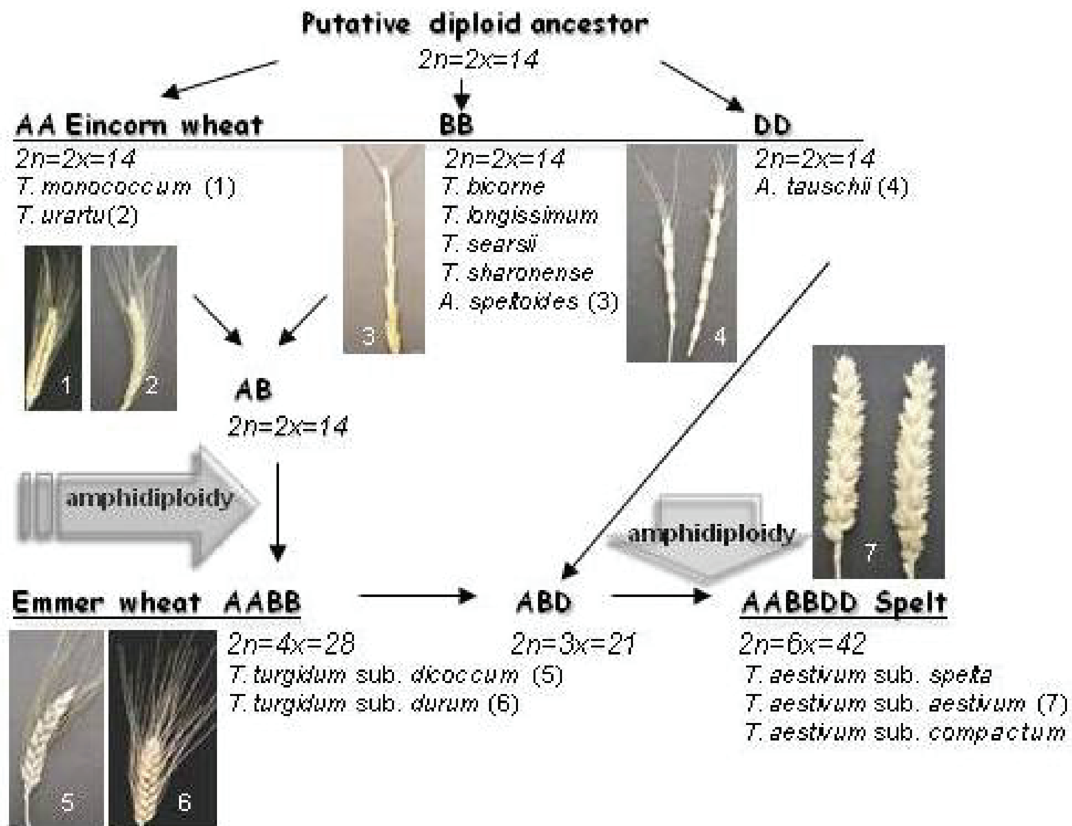

Figure 25 The evolution of the Triticeae species from a common ancestor (modified from Biswas *et al*., 2008).

Appendix 2

The genetic relationships among cultivated species of *Brassica*

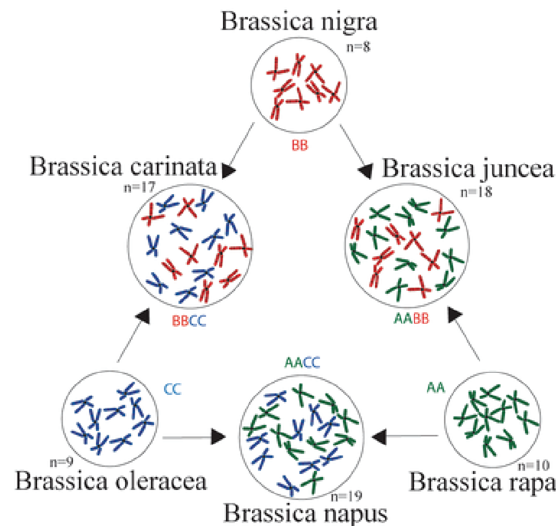

Figure 26 Triangle of U showing the genetic relationships between the six cultivated species of *Brassica* (http://blog.quantum-immortal.net/).

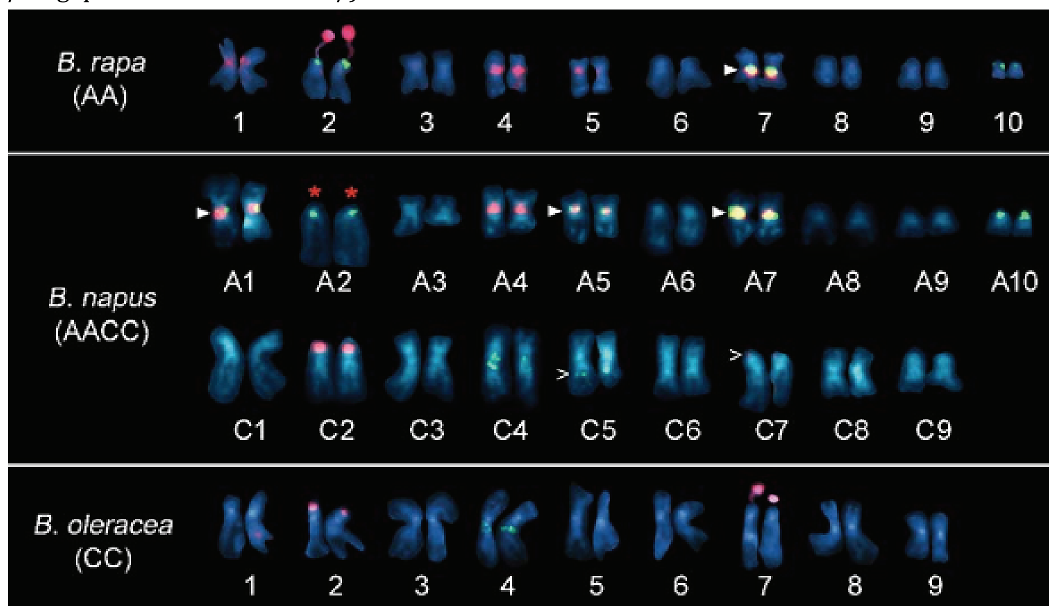

Figure 27 Karyotypes based on fluorescence in situ hybridization patterns with 5S (*green*) and 25S (*red*) rDNA probes and DAPI staining (*blue*), for *Brassica rapa* L., *B. oleracea* L. and their amphidiploid *B. napus* L. Closed arrowheads: co-localisation of 5S and 25S loci; open arrowheads: weak 5S locus and weak 25S locus on *B. napus* chromosomes C5 and C7, respectively. Red asterisks: position of large 25S rDNA locus located on satellite of B. napus chromosome A2, which in this spread was lost during chromosome preparation. The *B. napus* karyotype is divided into two sets of chromosomeswith differing chromatin condensation patterns resembling, respectively, those of *B. rapa* (a) and *B. oleracea* (c). Each *B. napus* chromosome is aligned and numbered in accordance with its putative homologue in the *B. rapa* or *B. oleracea* genome. (Snowdon *et al*. 2002).

Appendix 3

Isolation of total RNA by TRI Reagent® Solution (Ambion)

Description

TRI Reagent® Solution is a complete and ready-to-use reagent for the isolation of total RNA or the simultaneous isolation of RNA, DNA, and protein from diverse biological material (human, animal, plant, yeast, bacterial and viral origin) over ~ 5 mg tissue or 5 x 10^5 cultured cells. Immediate inhibition of RNase activity is provided by a combination of phenol and guanidine thiocyanate in a monophasic solution. A biological material is homogenized and lysed in TRI Reagent solution, and then separated into aqueous and organic phases by adding bromochloropropane (BCP) or chloroform and centrifuging. RNA partitions to the aqueous phase, DNA to the interphase, and proteins to the organic phase. Next the RNA is precipitated from the aqueous phase with isopropanol, and finally it is washed with ethanol and solubilized.

TRI Reagent® Solution - RNA Isolation Protocol

Homogenization

Grind tissue in liquid nitrogen in grinding mortar and pestle. Transfer to a 15 ml tube and leave the tubes at -80˚C.

Incubation

Remove your samples from the -80˚C freezer on dry ice or into liquid nitrogen and immediately pour preheated (60˚C) TRI Reagent solution (1 ml TRI Reagent solution per 100 mg tissue) into each tube. Mix (vortex) samples well and incubate the homogenate for 5 min at 60˚C in the fume hood.

Removing of Insoluable Material

Centrifuge samples at 3000 rpm at 4˚C for 20 min (increase speed if centrifuge and tubes allow it). Transfer supernatant to a new tube (15 ml).

Phase Separation

Add 200 µl of chloroform (without isoamyl alcohol) or 100 µl BCP per 1 ml of TRI Reagent solution and vortex for 15 sec. Leave tubes at room temp 2-3 min and centrifugate at 3000 rpm at 4˚C for 20 min.

RNA Precipitation

Transfer the aqueous phase (colorless top layer) to a fresh tube, add 0.5 volumes of isopropanol and 0.5 volumes of a high salt precipitation solution (e.g. 0.8 M sodium citrate and 1.2 M NaCl). Mix and leave at room temperature for 10 min. Centrifuge tubes at 10,000 x g at 4˚C for 15 min.

Wash

Carefully remove the supernatant without disturbing the pellet. Wash precipitated RNA forms a gel-like or white pellet with 1 ml of 75% ethanol per 1 ml TRI Reagent solution used for sample homogenization to each sample. Centrifuge at 10,000 x g at 4˚C for 10 min and remove the ethanol wash without disturbing the pellet. Briefly dry pellet (5-10 min; not longer).

RNA Resuspension

Resuspend pellet in 100 µl DEPC[1] H_2O and add RNase inhibitor (Promega). If necessary, increase the resuspension volume or incubate at 55-60˚C for 10 min to completely dissolve the pellet. Put an aliquot into the spectrophotometer to determine RNA concentration and quality.

Storing

Store at 4˚C for immediate analysis. For long-term storage, store at -70˚C or colder.

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1 Diethylpyrocarbonate (DEPC) is used to inactivate the RNase enzymes from water by the covalent modifications of the histidine residues. 1 l of water is usually treated with 1 ml of diethylpyrocarbonate for at least 1 hour at 37°C and then autoclaved (at least 15 min) to inactivate traces of DEPC. Inactivation of DEPC in this manner yields CO_2, H_2O and EtOH.

Appendix 4

RNA Cleanup (Qiagen)

Description and Basic Recommendations

This protocol can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or to desalt RNA samples. Buffer RLT and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

A maximum amount of starting material to be cleaned up in this protocol is 100 µg. This amount corresponds to the RNA binding capacity of the RNeasy spin column. Perform all steps of the procedure at room temperature. During the procedure, work quickly.

RNA Cleanup Protocol

Buffer RLT

Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix well.

Ethanol

Add 250 µl ethanol (96-100%) to the diluted RNA, and mix well by pipetting. Do not centrifugate. Continue immediately with next step.

Loading

Transfer the sample (700 µl)to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through.

Wash with RPE

Add 500 µl Buffer RPE[1] to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discsard the flow-through.

Wash with RPE

Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane to dry the RNeasy silica-gel membrane.

Extra centrifugation

Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

Elution

Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.

Repeat Elution

To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate. The yield will be 15-30% less than the yoeld obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

[1] Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

Appendix 5

DNA-*free*™ Kit (Ambion)

Description

Ambion's DNA-*free*™ DNase Treatment and Removal Reagents are designed to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample. Contaminating DNA is digested and the DNase is then removed rapidly and easily using a novel method which does not require phenol/chloroform extraction, alcohol precipitation, heating, or the addition of EDTA. In addition to removing the DNase enzyme, DNase Inactivation Reagent also removes divalent cations, such as magnesium and calcium, which can catalyze RNA degradation when RNA is heated with the samples. DNA-*free* treated RNA is suitable for endpoint or real-time RT-PCR, microarray analysis, and all other RNA analysis methods.

This protocol is designed to remove trace to moderate amounts of contaminating DNA (up to 50 μg DNA/ml RNA) from purified RNA to a level that is mathematically insignificant by RT-PCR. The recommended reaction size is 10-100 μl. A typical reaction is 50 μl.

DNA-*free*™ Kit Protocol

Add DNase Digestion Reagents

Add 0.1 volume 10× DNase I Buffer and 1 μl rDNase I to the RNA, and mix gently.

Incubate

Incubate at 37˚C for 30 min.

Add DNase Inactivation Reagent

Resuspend the DNase Inactivation Reagent by flicing or vortexing. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.

Incubate and Mix

Incubate 2 min at room temperature and mix the contents of the tube 2-3 times during the incubation period to redisper the DNase Inactivation Reagent.

Centrifuge and transfer RNA

Centrifuge at 10,000 x g for 2 min and carefully transfer the supernatant, which contains the RNA, into a fresh tube. Avoid introducing the sediment DNase Inactivation Reagent into solutions that may be used for downstream enzymatic reactions, because it can sequester divalent cations and change the buffer conditions.

Appendix 6

Agilent 2100 Bioanalyzer

The RNA molecule plays a critical role in transferring information encoded in the genome (DNA) to the many different forms of proteins. After extracting RNA from cells by various methods, scientists are provided with a direct measure of cellular activity using gene expression measurement techniques. Among these, real-time PCR and DNA microarrays are the most widely used techniques. For this reason is the integrity of RNA molecules paramount importance for experiments that try to reflect the snapshot of gene expression at the moment of RNA extraction. Historically was RNA integrity evaluated using agarose gel electrophoresis stained with ethidium bromide, which produces a certain banding pattern. Typically, gel images show two bands comprising the 28S and 18S ribosomal RNA (rRNA) species and other bands where smaller RNA species are located. RNA is considered of high quality when the ratio of 28S:18S bands is about 2.0 and higher. Since this approach relies on human interpretation of gel images, it is subjective, hardly comparable from one lab to another, and the resulting data cannot be processed digitally.

RNA 6000 Nano kit utilizes micro-fabricated chips with 12-wells requiring minimum sample consumption, in the low µl-range. The Agilent 2100 Bioanalyzer and its portfolio of Agilent RNA kits have become the industry-standard tool for RNA quality control, ensuring highest RNA sample integrity[1]. For plant cell or tissue samples, high quality total RNA should exhibit clear, sharp ribosomal RNA bands. The number of ribosomal bands can vary between species and tissue types. For example, photosynthetic tissue, such as leaf tissue, contains several smaller ribosomal RNAs from the chloroplast in addition to the cytosolic 25S and 18S rRNAs bands. Non-photosynthetic tissue, such as seed, or root tissue do not contain these extra plastid rRNA bands (for comparison seeFigure 29 and Figure 30). Quality plant total RNA should have sharp, distinct rRNA peaks. High baseline and indistinct peaks are indicative of RNA degradation. Messenger RNA from plants typically accounts for only 0.1% of the total RNA and is usually not seen in an electropherogram. The apparent integrity of a total RNA sample can vary with sample collection and processing, as well as inappropriate storage and sample handling.

[1] Agilent developed the algorithm called RNA integrity number (RIN), which standardized the assessment of RNA quality. After you analyze an RNA sample on the 2100 Bioanalyzer, the software automatically assigns an integrity number from 1 (totally degraded) to 10 (intact). In plant tissues containing chloroplast is not possible follow the RIN number for measurement of RNA integrity given the presence of other peaks.

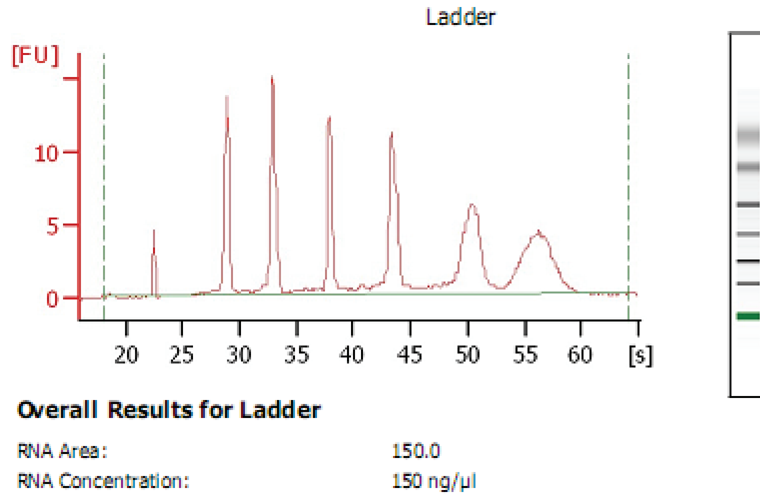


Figure 28 Electropherogram of the ladder for evaluation of the samples by Agilent 2100 bioanalyzer.

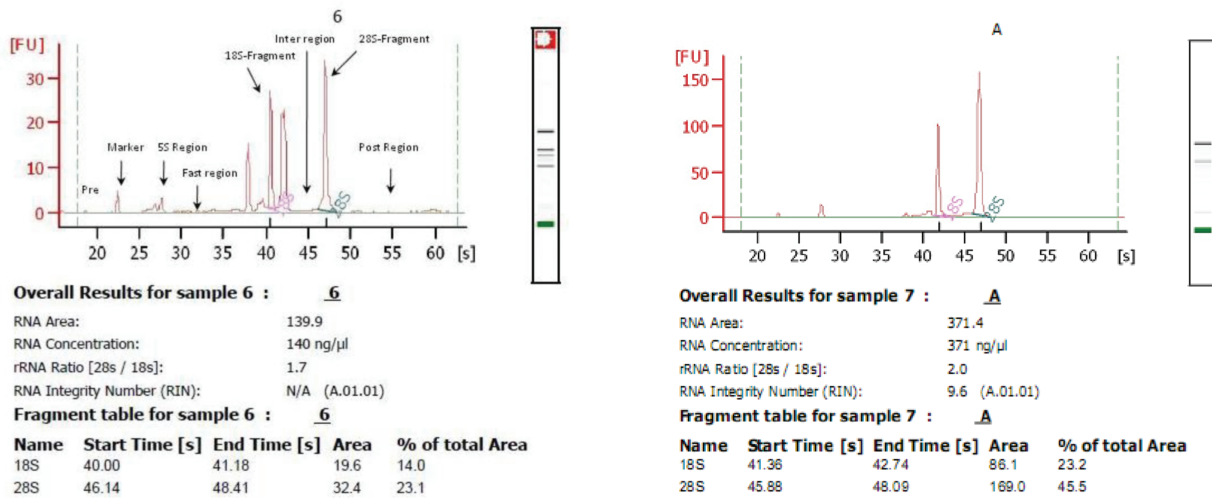


Figure 29 Comparison of segments of an electropherogram in different tissue types: sample 6 contains photosynthetic tissue (leaf tissue, which contains several smaller ribosomal RNAs from the chloroplast in addition to the cytosolic 25S and 18S rRNAs bands). Sample A is typical non-photosynthetic tissue (root tissue, which does not contain these extra plastid rRNA bands).

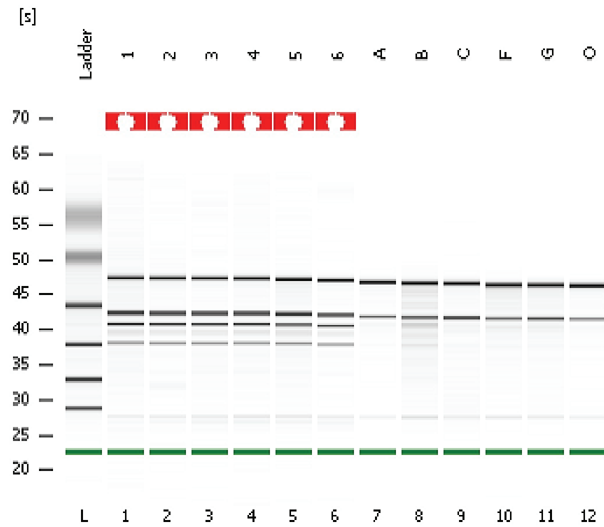


Figure 30 Electrophoresis summary from Agilent 2100 Bioanalyzer: ladder, first 6 samples are from photosynthetic tissue (leaves of *T. durum*), the samples from A to O are from the seeds of *T. durum* and do not contain on electrophoresis last two bands of chloroplast RNA. Red labeling means that RIN number was not available.

Agilent RNA Nano Assaz Protocol – Edition April 2007

Preparing the Gel

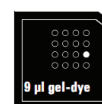
1 Pipette 550 µl of RNA 6000 Nano gel matrix (red•) into spin filter.
2 Centrifugate at 1500 g±20% for 10 minutes at room temperature.
3 aliquot 65 µl filtered gel into 0.5 ml Rnase-free microfuge tubes. Use filtered gel within 4 weeks.

Preparing the Gel-Dye Mix

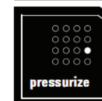
1 Allow the RNA 6000 Nano dye concentrate (blue •) to equilibrate to room temperature for 30 min.
2 Vortex RNA 6000 Nano dye concentrate (blue •) for 10 seconds, spin down and add 1 µl of dye into a 65 µl aliquot of filtered gel.
3 Vortex solution well. Spin tube at 13000 g for 10 min at room temperature. Use prepared gel-dye mix within one day.

Loading the Gel-Dye Mix

1 Put a new RNA 6000 Nano chip on the chip priming station.
2 Pipette 9.0 µl of gel-dye mix in the well marked G



3 Make sure that the plunger is positioned at 1 ml and then close the clip priming station



4 Press plunger until it is held by the clip.
5 Wait for exactly 30 seconds then release clip.
6 Wait for 5 seconds. Slowly pull back plunger to 1 ml position.
7 Open chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked **G**.

8 Discard the remaining gel-dye mix.

Loading the Agilent RNA 6000 Nano Marker

1 Pipette 5 µl of RNA 6000 Nano marker (green •) in all 12 sample wells and in the well marked .

Loading the Ladder and Samples

1 Pipette 1 µl of prepared ladder in well marked .
2 Pipette 1 µl of sample (before heat denature it for 2 min at 70 °C and immediately cool down the vial on ice for 5 min) in each of the 12 sample wells. Pipette 1 µl of RNA 6000 Nano Marker (green •) i each unused sample well.
3 Put the chip horizontally in the adapter of the IKA vortexer and vortex for 1 min at 2400 rpm.
4 Run the chip in the Agilent 2100 bioanalyzer within 5 min.

Appendix 7

Standart Reverse Transcription Protocol (Promega)

Prepare RNA Target and Primer (on ice)

1. For each 20µl reverse transcription (RT) reaction, combine:

Component	Volume
RNA template	up to 1µg
primer	20pmol or 0.5µg
nuclease-free water to a final volume of	**5µl**

2. Incubate at 70˚C for 5 minutes.
3. Quick-chill at 4˚C for 5 minutes and hold on ice.

Prepare Reverse Transcription Mix

1. For each 20µl reverse transcription (RT) reaction, combine:

Component	Concentration	Final Volume	Final Concentration
nuclease-free water		Xµl	
ImProm-II™	5× Reaction Buffer	4µl	1×
$MgCl_2$	25mM	2.4µl	3mM
dNTP mix	10mM each dNTP	1µl	0.5mM
Vortex the mixture and add:			
Improm- II™ Reverse Transcriptase		1 µl	
Final volume RT MIX per 20 µl reaction		**15 µl**	

2. Vortex gently to mix.
3. Dispense 15 µl aliquots into reaction tubes.

Add Template and Primers to the reaction Mix

Foe each individual reaction, add 5 µl of the appropriate Template and Primer mix to the 15 µl reverse transcription mix.

Reverse Transcription

1. Anneal at 25˚C for 5 minutes.
2. Extended the first strand for 60 minutes at 42˚C.
3. Heat-inactivate the ImProm-II™ Reverse Transcriptase by incubating at 70˚C for 15 minutes.
4. Analyze concentration of cDNA and store frozen.

Appendix 8

Quibit® Fluorometer (Invitrogen)

The Quibit® Quantitation Platform is highly sensitive fluorescence-based quantitation assays with fluorometer. The Quant-iT™ assay kits use state-of-the-art dyes selective for dsDNA, RNA, and protein and that is why is without risk about contaminants in the sample affecting the quantitation. The Qubit® fluorometer uses the very latest in illumination and detection technology for the highest sensitivity, it is possible to use as little as 1 µl of sample and still get great results, even with very dilute samples.

Critical Quant-iT™ Assay Considerations

Assay Temperature

The Quant-iT™ assays were designed to be performed at room temperature (22–28°C), as temperature fluctuations can influence the accuracy of the assay. To minimize temperature fluctuations, store all kit reagents at room temperature and insert all assay tubes into the Qubit® fluorometer only for as much time as it takes for the instrument to measure the fluorescence, as the Qubit® fluorometer can raise the temperature of the assay solution significantly, even over a period of a few minutes. Do not hold the assay tubes in your hand before reading, as this will warm the solution and result in a low reading.

Incubation Time

To allow the Quant-iT™ assay to reach maximum fluorescence, incubate the tubes for the DNA and RNA assays for 2 minutes after mixing the sample or standard with the working solution. After this incubation period, the fluorescence signal is stable for 3 hours at room temperature, for all nucleic acid assays except the Quant-iT™ ssDNA assay, which is stable for up to 30 minutes. For greatest accuracy of the protein assay, the incubation time of the samples should be within 10 minutes of the incubation time of the standards.

Protocol:

(for pictures see Figure 31)

1. Set up your tubes: two tubes for the standards and one tube for each of the samples.
2. Make the Quant-iT™ Working Solution by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ bufer. 200 µl of Working Solution are required for each sample and standard.
3. Prepare Assay Tubes according to the picture below.
4. Vortex all tubes for 2–3 seconds.
5. Incubate the tubes for 2 minutes at room temperature.
6. Read tubes in Qubit™ fluorometer.
7. Multiply by the dilution factor to determine concentration of your original sample.

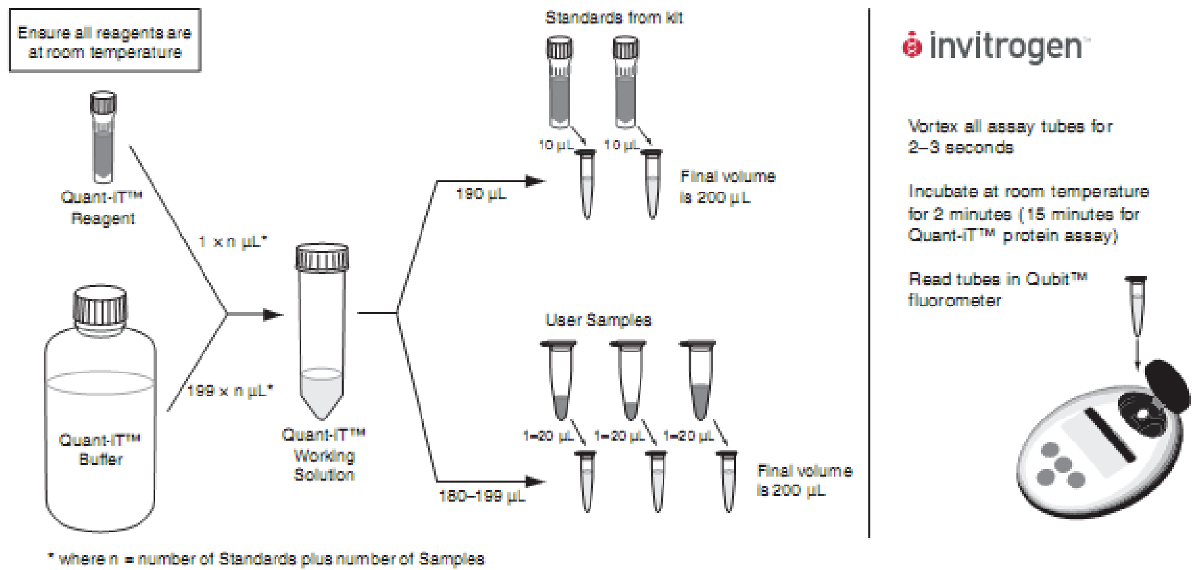


Figure 31 The Quibit® Quantitation.

Appendix 9

Primer Designing

How to proceed:

- Based on the different FC threshold (see Figure 10) between two wheat cultivars and interesting annotation associated with heat/drought stress, Affymetrix Probe Sets presenting gene of interest were selected.
- Sequences of this Probe Sets were found in Plant Expression Database – PLEXdb (http://www.plexdb.org/index.php).
- At least two consensus sequences of the members of the Probe Set were copied in file format text. The first and last sequences of the Probe set served to indicate the section for primer designing. These sequences were used moreover for verify that all operations were carried out properly.
- Obtained overall length sequences were compared with others homologous sequences presented as Plant Transcript Assemblies (represent a non-redundant set of transcripts through clustering and assembly of full-length and partial cDNAs, Expressed Sequence Tags (ESTs) and known mRNAs excluding the predicted CDS sequences derived from genome sequencing projects) producing High-scoring Segment Pairs in TIGR Plant Transcript Assembly BLAST Server (http://blast.jcvi.org/euk-blast/plantta_blast.cgi), which were published until now. The sequences with a high degree of similarity to the sequence of Probe Set were copied as a text format file in successive manner.
- After multiple alignment analysis of homologous sequences using the software BioEdit or Vector NTI Advance 11, have been identified output region with no homology to other sequences of wheat, specially in its 3' UTR region which was used for gene markers designing with Primer Express 3.0 program from Applied Biosystem imposing the following stringent criteria (see bellow Primer Express). In some cases were primer pairs modified manually or by using of Primer3 software v. 0.4.0 (http://frodo.wi.mit.edu/primer3/).
- Designed primer pairs were checked *in silico* (TIGR) to ensure their specificity and uniqueness of the gene of interest.

Primer Express 3.0 (Applied Biosystem)

For primer designing was function of TaqMan® RGB Quantification from software Primer Express 3.0 (Applied Biosystem) used (Table 12). No probes were selected and used. This application automatically generates primer pairs with specific properties for use in the qRT-PCR. It is essential that the primer pair formed amplicon 60-120bp with Tm = 60 °C ± 1 °C, because the universal cycle of qRT-PCR provides annealing and extension takes place in a total of 1 minute and 60 °C. In some cases were primer pairs modified manually or by using of software Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/).

Table 12 Primer Express 3.0 (Applied Biosystem) condition.

Parameters	Value	Parameters	Value
Primer Tm		Primer Secondary Structure	
Min Primer Tm	58	Max Primer Consec Base Pair	4
Max Primer Tm	60	Max Primer Total Base Pair	8
Max Difference in Tm of Two Primers	2	Primer Site Uniqueness	
Primer GC Content		Max % Match in Primer	75
Min Primer %GC Content	30	Max Consec Match in Primer	9
Max Primer %GC Content	80	Max 3' Consec Match in Primer	7
Max Primer 3' GC's	2	Amplicon	
Primer 3' End Length	5	Min Amplified Region Tm	0
Primer 3' GC Clamp Residues	0	Max Amplified Region Tm	85
Primer Length		Min Amplified Region Length	60
Min Primer Length	9	Max Amplified Region Length	120
Max Primer Length	40	General	
Optimal Primer Length	20	Max Primers	50
Primer Composition			
Max Primer G Repeats	3		
Max Num Ambig Residues in Primer	0		

Appendix 10

Preparing Samples for Sequencing

PCR Protocol

Component	Concentration	Final Volume	Final Concentration
Buffer	5×	4μl	1×
DMSO	100%	1μl	5%
$MgCL_2$	25mM/μl	1.2μl	1.5mM
dNTP	10mM/μl	0.5μl	0.25mM
primer F	10μM/μl	1μl	0.5μM
primer R	10μM/μl	1μl	0.5μM
Taq	5U/μl	0.2μl	1U
cDNA	2ng/μl	3μl	6ng
H_2O		8.1μl	
		20μl	

Amplification:
5min at 94˚C
35 cycles of:

30 s at 94˚C
30 s at 58˚C
1 min at 72˚C

5min at 72˚C
∞ min at 4˚C

Separation and Purification of PCR Products by 2% Agarose Gel Electrophoresis

Load the PCR products into the gel and than excise DNA bands from gel.

Wizard® SV Gel Clean-Up System (Promega)

Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10μl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50-65˚C until gel slice is completely dissolved.

Binding of DNA

4. Insert SV Minicolumn into Collection Tube.
5. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
6. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

7. Add 700μl Membrane Wash Solution (ethanol added). Centrifugate at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
8. Repeat Step 7 with 500μl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5

minutes.
9. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

10. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
11. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Cenrifuge at 16,000 × *g* for 1 minute.
12. discard Minicolumn and store DNA at 4˚C or -20˚C.

Quantification by 2% Agarose Gel Electrophoresis

For quantification were three dilution series of the Lambda prepared and PCR products were quantified by using Quantity One® software (Bio-Rad).

Big Dye Sequencing reaction

Component		Volume
Big Dye		0.3µl
Buffer 5×		2µl
Primer (F or R) 10µM		0.8µl
Template	2ng for each 100bp	Xµl
H_2O		Xµl
		10µl

Amplification:

1min at 96˚C
30 cycles of:

10 s at 96˚C
15 s at 50˚C
4 min at 60˚C

∞ min at 4˚C

Purification to remove residual incorporated dye primers

1. For each samples prepare and add 80µl of the following mixture:
   3µl Na acetate 3M, pH 4.6
   62.5µl ethanol (95% from freezer)
   14.5µl H_2O
2. Invert 3-times the tube and then store it at -80˚C for 30 min.
3. Centrifuge for 30 min at 3,000 × *g*
4. Remove the supernatant carefully (without touching the pellet on the bottom of the tube), invert the tube and spin it for 1 min at 50 × *g*.
5. Wash the pellet with 150µl of 70% ethanol from freezer (convert 3-times) and spin the tube at 3,000 × *g* for 15 min and repeat step 4.
6. Dry the pellet in room temperature for 15 min (covered with filter paper).
7. Resuspend the pellet in 20µl of sterile H_2O.

Appendix 11

Quantitative RT-PCR (qRT-PCR) description and dissociation curves troubleshooting

QRT-PCR is currently one of the most powerful and sensitive techniques for analyzing gene expression and, among its several applications, it is often used for validating output data produced by micro- and macro- arrays of whole-genomes and as a primary source for detecting specific gene expression patterns.

Reliable quantification by qRT-PCR analysis of gene expression levels requires the standardization and optimalization of several parameters, such us: amount of initial sample, RNA recovery, storage, purity and integrity, enzymatic efficiencies of cDNA synthesis and PCR amplification.

Direct detection of PCR product is monitored by measuring the increase in fluorescence caused by the binding SYBR Green to dsDNA. The passive reference is ROX, a dye included in the 10× SYBR Green PCR Buffer that does not participate in the PCR amplification. The passive reference provides an internal reference to which the SYBR Green-dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume. Dividing the emission intensity of SYBR Green by the emission intensity of the passive reference is possible obtain a ratio defined as the R_n (normalized reporter). Then $\Delta R_n = (R_n^+)-(R_n^-)$, where R_n^+ is R_n value of a reaction containing all components including the template and R_n^- is R_n value of an unreacted sample. ΔR_n indicates the magnitude of the signal generated by the given set of PCR conditions.

From several proposed methods is normalization of gene expression data using of internal control genes (reference genes) one of the most common way, which exhibit highly uniform expression throughout experimental conditions and reduce possible errors generated in the quantification of gene expression, which is obtained by comparing the expression levels in the analysed samples of the gene of interest and of stable constitutive control genes. For this reason the success of this procedure depends on the choice of adequate control genes, which ideally would be those showing stable expression under various experimental conditions, development stages and in different types of tissue. Until recently the most well-known reference genes used for normalization in gene expression analysis in different species are those encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GTP-binding proteins, SAND proteins, actins, ubiquitins, tubulins, cyclophilins, elongation factors and 18S rRNA, which are involved in basic cellular processes. These genes have commonly been employed in traditional methods of gene expression detection, such as nothern blot, Rnase protection test and conventional semi-quantitative RT-PCR (Paolacci *et al.*, 2009). Unfortunately, several reports have shown that frequently the most widely used House Keeping Genes (HKGs) are not reliable controls, since their expression levels are also regulated and vary considerably with experimental conditions, and it would be essential a preliminary evaluation for identifying the most stable HKGs in each species.

On the contrary, there exists few expression analysis studies of HKGs in different plant species and experimental conditions by using the same well-known reference

genes. However the fact, that currently a novel set of reference genes has been identified in rice (Narsai *et al*., 2010, Jain, 2009), wheat (Aprile *et al*., 2009) and *Arabidopsis* (Czechowski *et al*., 2005) by genome-wide analysis of the whole genome using microarray data indicate that many new reference genes outperforming the traditional ones in terms of expression stability can be found by different approaches.

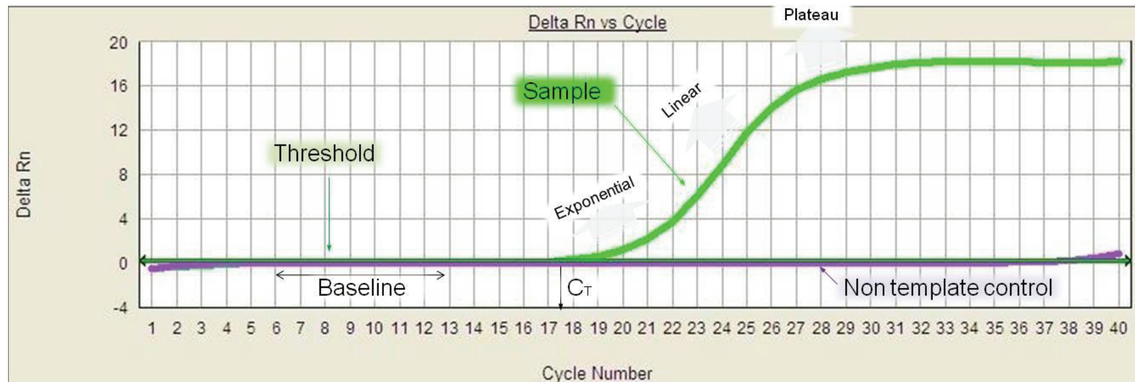


Figure 32 Real Time PCR Output. The threshold cycle or C_T value is the cycle number at which amplification curve crosses threshold detecting statistically significant increase in R_n. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor. Real-Time PCR can detect product in exponential phase allowing more precise quantification. Amplification is composed of successive phases: exponential, linear and plateau phase which detect End-Point PCR.

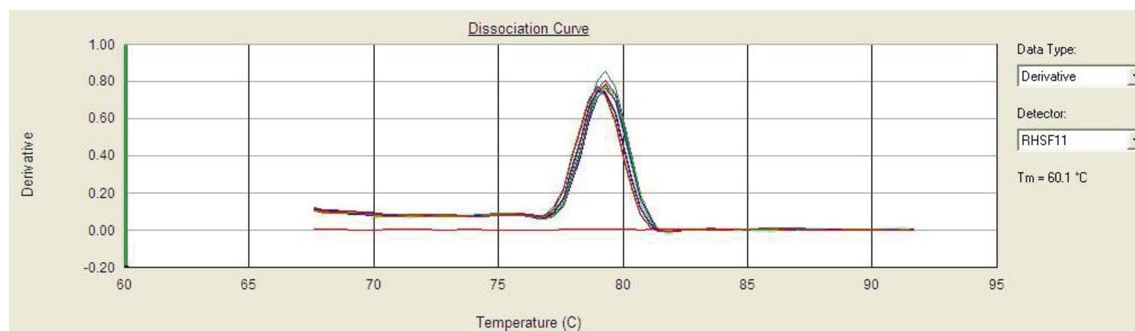


Figure 33 RT-PCR dissociation curve of *RHSF11* gene with generated single-peak melting curves signaling single amplicons and primer specificity. The flat red line represents blank (non-template control).

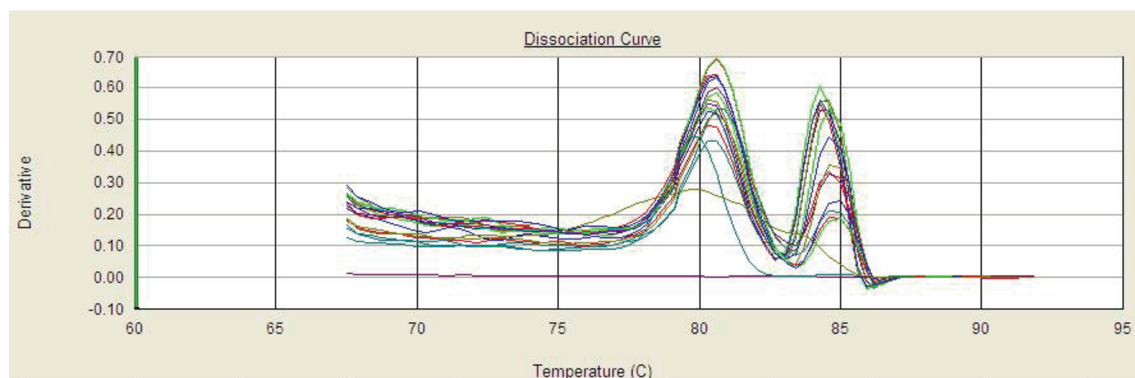


Figure 34 RT-PCR dissociation curve generated double-peak melting curves. Primer pairs which were used in this analysis are unsuitable for further using and should be redesigned.

Table 13 qRT-PCR of reference genes. Genes with the lowest variation between different cultivars, drought-stress condition and development stage have the most stable expression and therefore were selected as ideal reference genes. Each series (of different RSWC) is composed mean of 4 samples (its three technical repetitions) compound Ofanto stressed, Cappelli stressed and their control plants.

	Series (RSWC%)	Mean Ct	Ct min	Ct max	Δct	Standard deviation	Standard error	CV
cyclophilin	**78/79**	21.81	21.18	22.27	1.090	± 0.379	± 0.109	0.017
	49	21.14	20.57	21.74	1.170	± 0.328	± 0.095	0.016
	34/35	20.98	20.60	21.28	0.680	± 0.203	± 0.059	0.010
	27/28	20.41	19.43	21.34	1.910	± 0.839	± 0.242	0.041
	total	21.084	19.43	22.27	2.840	± 0.700	± 0.101	0.033
NADH	**78/79**	20.55	19.95	21.15	1.200	± 0.394	± 0.114	0.019
	49	20.69	20.27	21.03	0.760	± 0.287	± 0.083	0.014
	34/35	20.37	19.77	21.03	1.260	± 0.395	± 0.114	0.019
	27/28	19.48	18.74	19.75	1.010	± 0.341	± 0.098	0.018
	total	20.27	18.74	21.15	2.410	± 0.588	± 0.085	0.029
polyubiquitin	**78/79**	21.70	21.08	22.12	1.040	± 0.380	± 0.110	0.018
	49	21.50	21.08	22.00	0.920	± 0.274	± 0.079	0.013
	34/35	21.31	19.92	22.61	2.690	± 0.968	± 0.279	0.045
	27/28	21.02	20.27	21.47	1.200	± 0.422	± 0.122	0.020
	total	21.382	19.92	22.61	2.690	± 0.612	± 0.088	0.029
TaRP15	**78/79**	21.54	21.07	22.05	0.980	± 0.324	± 0.093	0.015
	49	20.87	19.41	21.63	2.220	± 0.826	± 0.239	0.040
	34/35	21.05	20.12	21.50	1.380	± 0.556	± 0.161	0.026
	27/28	20.94	20.30	21.90	1.600	± 0.590	± 0.170	0.028
	total	21.097	19.41	22.05	2.640	± 0.639	± 0.092	0.030
TaRPII36	**78/79**	25.72	24.59	26.47	1.880	± 0.632	± 0.182	0.025
	49	25.43	24.94	25.93	0.990	± 0.324	± 0.094	0.013
	34/35	25.42	24.86	26.06	1.200	± 0.401	± 0.116	0.016
	27/28	24.86	23.64	26.06	2.420	± 0.962	± 0.278	0.039
	total	25.355	23.64	26.47	2.830	± 0.687	± 0.099	0.027
TaSnRK1	**78/79**	22.09	21.60	22.47	0.870	± 0.290	± 0.084	0.013
	49	20.68	16.31	22.44	6.130	± 2.598	± 0.750	0.126
	34/35	20.38	15.67	22.35	6.680	± 2.830	± 0.817	0.139
	27/28	21.85	21.14	22.45	1.310	± 0.439	± 0.127	0.020
	total	21.250	15.67	22.47	6.800	± 2.017	± 0.291	0.095
TIM 17	**78/79**	19.38	18.90	19.72	0.820	± 0.211	± 0.061	0.011
	49	19.61	19.23	20.14	0.910	± 0.307	± 0.089	0.016
	34/35	19.55	18.70	20.17	1.470	± 0.557	± 0.161	0.028
	27/28	19.05	18.75	19.27	0.520	± 0.196	± 0.057	0.010
	total	19.396	18.70	20.17	1.470	± 0.404	± 0.058	0.021

Publications

Havlíčková, L., Čurn, V., Kukolíková, B., 2009. Marker asisted selection in hybrid breeding of oil seed rape. Úroda 12, scietific annex, 23-29.

Havlíčková, L., Marè, C., Rizza, F., Aprile, A., Borrelli, G., Panna, R., Perrotta, C., De Bellis, L., Mazzucotelli, E., Cattivelli, L., Čurn V., 2010. Validation of microarray analysis and identification of differentially expressed genes in two durum wheat cultivars with contrasting response to drought stress. Úroda 12, scientific annex, 163 – 166.

Participation in scientific congresses, conferences, workshops etc. (lecture/poster)

Havlíčková, L., Čurn, V., Kukolíková, B., 2009. Marker asisted selection in hybrid breeding of oil seed rape. International conference „Aktuální poznatky v pěstování, šlechtění, ochraně rostlin a zpracování produktů", November 2009 Brno, Czech Republic – lecture, poster

Havlíčková, L., Čurn, V., Kukolíková, B., 2010. The development of specific molecular marker for detection of self-incompatible plants a powerful tool in *Brassica napus* hybrid breeding. XXX Konferencja Naukowa „Rośliny Oleiste", March 2010 Poznań, Poland – poster

Aprile, A., Havlickova, L., Mare, C., Borrelli, G.M., Panna, R., Perrotta, C., Bellis, L., Mazzucotelli, E., Rizza, F., , Cattivelli, L. 2010. Transcriptomic Analysis of Drought and Heat Responses in Durum Wheat. 54° Annual Congress, Italian Society Agricultural Genetics (SIGA), September 2010 Matera, Italy - poster

Havlíčková, L., Marè, C., Rizza, F., Aprile, A., Borrelli, G., Panna, R., Perrotta, C., De Bellis, L., Mazzucotelli, E., Cattivelli, L., Čurn, V., 2010. Identification of differentially expressed genes in two durum wheat cultivars with contrasting response to drought stress. International conference „Aktuální poznatky v pěstování, šlechtění, ochraně rostlin a zpracování produktů", November 2010 Brno, Czech Republic - poster

Havlíčková, L., Marè, C., Rizza, F., Aprile, A., Borrelli, G., Panna, R., Perrotta, C., De Bellis, L., Mazzucotelli, E., Krajníková, E., Cattivelli, L., Čurn, V., 2010. Identification of differentially expressed genes in response to drought stress for eQTLs mapping in durum wheat. Olomouc Biotech 2011, June 2011 Olomouc, Plant Biotechnology: Green for Food, Czech Republic - poster

Čurn, V., Havlíčková, L., Kukolíková, B., Šimáčková, K., Vondrášková, E., 2011. Molecular markers: Tools for detection of self-incompatible plants in oil seed rape hybrid breeding. 9th Plant Genomics European Meetings (Plant GEM), May 2011 Istanbul, Turkey - poster

Marè, C., Havlickova, L., Rizza, F., Aprile, A., Borrelli, G.M., Panna, R., Mastrangelo, A.M., Perrotta, C., De Bellis, L., Cattivelli, L., 2011. Transcriptomic analysis of drought and heat responses in durum wheat and eQTLs mapping to identify the loci controlling the molecular response to drought. Italian Society Agricultural Genetics (SIGA), September 2011, Assisi, Italy – poster

Havlíčková, L., Marè, C., Rizza, F., Aprile, A., Borrelli, G.M, Panna, R., Mastrangelo, A.M., Perrotta, C., De Bellis, L., Mazzucotelli, E., Krajníková, E., Čurn, V., Cattivelli, L. 2011. Identification of differentially expressed genes in response to drought stress for eQTLs mapping in durum wheat. 9th Plant Genomics European Meetings (Plant GEM), May 2011 Istanbul, Turkey - poster

Havlickova, L., Kukolikova, B., Curn, V., 2011. The development of molecular marker for detection of self-incompatible plants: a powerful tool in *Brassica napus* hybrid breeding. 13th International Rapeseed Congress, June 2011 Praha, Czech Republic – poster

Grant support

This study was supported by:

GA ČR 521/08/H042 granted to Vladislav Čurn
GAJU 032/2010/Z to Lenka Havlíčková
GAJU 064/2010/Z to Vladislav Čurn
IG 05/08 to Lenka Havlíčková
IG 05/09 to Lenka Havlíčková
NAZV 1G46061 to Vladislav Čurn
NAZV QI111A075 to Vladislav Čurn
MSM 6007665806 to Vladislav Čurn