An integrated approach to identify stress-related regulatory genes in cork oak

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Abstract

Cork oak (*Quercus suber*) is an evergreen tree of the *Fagaceae* family with great economic impact in Portugal, due to cork (outer layer of the bark). In recent decades, there has been a decline in population due to a number of biological factors (e.g. *Phythophthora cinnamomi*, insects) and environmental factors (e.g. heat waves, drought and severe cold in winter). The lack of genetic information on cork oak has hampered the understanding of the basic mechanisms regulating stress response and adaptation. The present work is integrated within a project (SuberStress), which aims to identify new candidate genes and regulatory pathways involved in the crosstalk between different stress conditions. In this work we extracted RNA samples from leaves previously exposed to heat and cold stress assays to prepare the non-normalized EST libraries (through 454- pyrosequencing). An *in silico* analysis of these EST libraries together with previously prepared libraries for drought stress and *P. cinnamomi* infection generated a list of candidate genes showing to be differentially regulated by several stresses (this work was made by another member of the team). To validate the expression of these candidate genes we performed another heat stress assay. Physiological data were recorded and leaf samples were collected. Total RNA was extracted from several samples (for control, shock and acclimation) and gene expression analysis was conducted by RT-PCR.

During the assay the plants showed an active response to high temperatures using transpiration to cool the leaves, and the efficiency of photosystem II was maintained with the increasing temperature. These data may indicate an adaptive mechanism to heat. Of the 16 candidates analyzed by RT-PCR, 13 have shown expression patterns in response to heat/cold stresses consistent with the *in silico* analysis. The expression of these genes will be also validated for the remaining stresses and the most interesting genes will be functionally characterized.

Keywords: *Quercus suber*, cork oak, thermal stress, stress response, regulatory genes

Sumário

O sobreiro (*Quercus suber*) é uma árvore da família *Fagaceae*, com grande impacto económico (bem como ecológico) em Portugal, devido à cortiça (camada externa do tronco). Nas últimas décadas, tem havido um declínio na população de sobreiros devido a uma série de factores biológicos (por exemplo, infecção por fungos, como *Phythophthora cinnamomi*, e insectos) e factores ambientais (por exemplo, ondas de calor, seca e ondas de frio intenso no inverno). A falta de informação sobre a genética do sobreiro tem dificultado a compreensão dos mecanismos de regulação e adaptação envolvidos na resposta ao *stress*. O presente trabalho está integrado num projecto (*SuberStress*) que visa identificar novos genes candidatos, e vias regulatórias, que possam estar envolvidos no *crosstalk* entre diferentes condições de *stress* biótico e abiótico. Neste trabalho, amostras de RNA foram extraídas de folhas colectadas em ensaios prévios de calor e de frio; o RNA extraído foi utilizado para preparar bibliotecas não-normalizadas de transcritos (EST), por 454-*pyrosequencing* (BIOCANT). Da análise *in silico* das bibliotecas não-normalizadas de transcritos de *stress* térmico, com as bibliotecas previamente preparados para os stresses de secura e infecção por *P. cinnamomi*, foi obtido um grupo de genes candidatos (respondendo a pelo menos dois *stresses*; este trabalho foi desenvolvido por outro membro do projecto). Para validar a expressão dos genes candidatos foi realizado um novo ensaio de *stress* por calor. Foram recolhidos dados fisiológicos e amostras biológicas (folhas). Durante o ensaio, e estando em condições de rega abundante, as plantas apresentaram uma resposta activa às altas temperaturas utilizando a transpiração para arrefecer as folhas. A eficiência do fotossistema II, manteve-se durante o ensaio, tanto para a situação de choque como de aclimatação. Estes dados podem indicar um mecanismo de adaptação ao calor.

O RNA total foi extraído a partir de várias amostras (para controlo, choque e aclimatação, a calor e a frio). Com esse RNA procedeu-se à análise dos padrões de expressão dos genes candidatos. Dos 16 candidatos analisados por RT-PCR, 13 mostraram padrões de expressão em resposta ao calor/frio consistentes com os padrões previstos na análise *in silico*. Estes genes serão igualmente validados para outros *stresses* (secura, salinidade, infecção) e os genes mais interessantes serão caracterizados funcionalmente.

Palavras-chave: sobreiro, *Quercus suber*, stress térmico, mecanismo de resposta ao stress, genes regulatórios

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List of Abbreviations

µmol	Micro mole
%	Percentage
^{o}C	Degree centigrade
A_n	Net photosynthesis
C_i	Intercellular CO_2 concentration
cm	Centimeter
CO_2	Carbon dioxide
COEC	Cork oak EST consortium
DB	Data base
EUFORGEN	EUropean FORest GENetic resources programme
g	Gram
g_s	Stomatal conductance
h	Hours
H_2O	Water
KOAc	Potassium acetate
L	Liter
M	Molar
m	Meter
Max	Maximum
mg	Milligram
min	Minutes
Min	Minimum
mL	Milliliter
mm	Millimeter
mol	Mole
NaOAc	Sodium acetate
nm	Nanometer
ng	Nanogram
O_2	Oxygen
PPFD	Photosynthetic photon flux density

ppm	Parts *per* million
PSII	Photosystem II
PVP	Polyvinylpyrrolidone
qPCR	Real Time - Polymerase Chain Reaction
RH	Relative humidity
rpm	Rotations *per* minute
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
DNA	Desoxyribonucleic acid
rt	Room temperature
RWC	Relative water content
s	Second
SD	Standard deviation
SE	Standard error
SNPs	Single Nucleotide Polymorphisms
V	Volume
Φ_{PSII}	Photosystem II efficiency

Chapter 1. Introduction

1. *Quercus suber*: biology and species distribution

Quercus suber, also known as cork oak (Figure 1), is a national symbol in Portugal (Pereira and Caldeira, 2011). It is an evergreen tree from the *Fagaceae* family (Manos *et al.*, 2001), adapted to the Mediterranean climate (Manos *et al.*, 2001; Lorenzo *et al.*, 2009; Gil and Varela, 2008; Aranda *et al.*, 2005) and with a great economical importance for the Iberian Peninsula, due to its characteristic thick suberin bark named cork (Chaves *et al.*, 2011b; Pereira and Caldeira, 2011; Fernandes, 2011).

Figure 1: Cork oak tree (http://www.amorimcork.com/pt/).

The *Fagaceae* family belongs to the *Fagales* order (dicotyledonous woody flowering plants) and includes nine genera, being the most important: *Fagus* L. (beeches), *Castanea* L. (chestnuts), and *Quercus* L. (oaks). They are monoecious trees and shrubs with a characteristic seed called acorn (Figure 2). They can be evergreen or deciduous (Manos *et al.*, 2001, Gil and Varela, 2008).

Figure 2: Cork oak acorn (http://www.amorimcork.com/pt/).

This family is economically important worldwide, mainly for timber (floor, furniture, and wine barrels), food (chestnuts, acorns) and cork (wine bottle stoppers, isolation, clothes,

shoes, belts, bags). Cork has a great economical importance in Portugal. This country exports the largest percentage of cork in the world (> 40%), representing 3% of country's Gross National Product (Fernandes, 2011; http://www.amorimcork.com/pt/natural-cork/what-and-why/).

The cork oak is a medium-sized tree, which can grow up to 20m, with a thick trunk and a large leaf canopy. This species, like other oaks, have a long life cycle (an average of 150-200 years). It is wind pollinated and has separated male and female flowers on the same plant. Acorn maturation can be annual or biennial (Gil and Varela, 2008). The leaves are long (4-7cm), thick and toothed, with a dark-green tone (Figure 3) in the adaxial side and a lighter tone on the abaxial side (with the presence of stomata and star-shaped thricomes) (Gil and Varela, 2008).

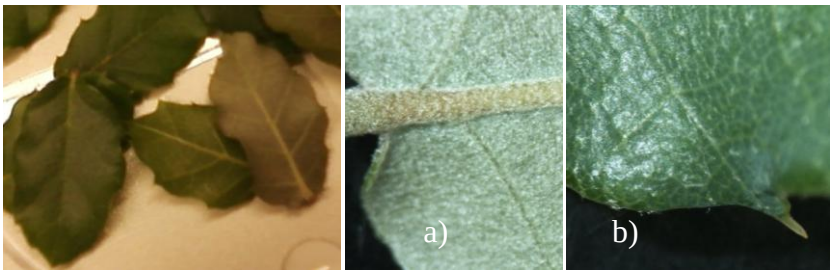


Figure 3: Fully expanded cork oak leaves. a) abaxial side of the leave; b) adaxial side of the leave.

Species from the *Fagaceae* family dominate the forests in temperate and seasonally dry regions of the Northern Hemisphere. They can also be found in tropical Southeast Asia (Manos *et al.*, 2001, Lorenzo *et al.*, 2009). Cork oak is distributed in the center and western Mediterranean basin (Figure 4). It is widespread in the Iberian Peninsula (Portugal and Spain) but can also be found in France, Italy, North Africa (Algeria, Tunisia and Morocco) and Sardinia, Corsica, Sicily, Majorca and Minorca Islands (Aranda, *et al.*, 2005, Gil and Varela, 2008, Chaves *et al.*, 2011b). This region has temperate average temperatures and low precipitation, with a season of warm to hot and dry summer and cold (but not freezing) and wet winter (Pinto *et al.*, 2011; http://gimcw.org/climate/characteristics.cfm).

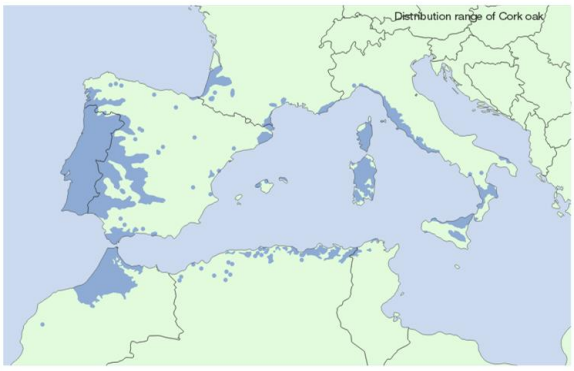


Figure 4: The Cork oak distribution in Mediterranean basin, represented in dark blue (Gil and Varela, 2008).

In Portugal, cork oaks can be found in three tree stands: *montados*, *sobreirais* or randomly scattered trees. *Montados* are an important ecosystem providing food and shelter for animals (wild and domestic) as well as participating in the carbon cycle and soil enrichment (Pereira and Caldeira, 2011; Gil and Varela, 2008, Vogiatzakis and Careddu, 2003). The *sobreirais* are cork oak forests with close canopy stands, generally in hill areas that cannot be cultivated; other trees can be present, however this ecosystem has predominance in cork oak trees, due to it's resistance to fires, that selectively removes other tree species (Pereira and Caldeira, 2011).

2. Environmental Challenges

Trees living in the Mediterranean climate must be adapted to withstand the hot and dry summers and the mild to cold and rainy winters (with a large temperature drop at nighttime) (Aranda, *et al.*, 2005, Gil and Varela, 2008) (Figure 5). Experts predict that in the next decades the climate changes in this region will have an increasing negative effect in plants, with the increasing frequency in heat and cold shock waves and changes in rainfall. These climate changes can lead to thermal stress in plants, as well as drought stress and an increase in summer fires. This can lead to changes in the plant metabolism and growth, as well as enhance the onset of diseases (Koskela *et al.*, 2007; Pereira and Caldeira, 2011; Chaves *et al.*, 2011b, Schaffhauser *et al.*, 2011, Vogiatzakis and Careddu, 2003, Correia *et al.*, 2011).

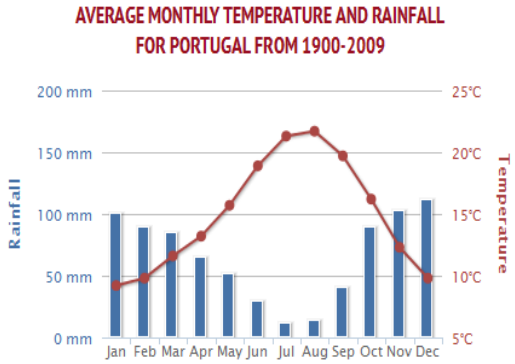


Figure 5: Mean historical monthly temperatures and rainfall for Portugal during 1900-2009 time periods; data collected by CRU (Climatic Research Unit, University of East Anglia, UK) (http://sdwebx.worldbank.org/climateportal/index.cfm).

Heat stress can have a devastating effect in plant metabolism, affecting cellular homeostasis and major physiological processes (e.g. photosynthesis). In its turn, the cold stress, specially the chilling shock (below 15ºC), decreases enzymatic activity and affects carbohydrate metabolism and photosynthetic components (Correia *et al.*, 2011; Correia *et al.*, 2012; Chaves *et al.*, 2011a). However, the effect of the stress depends on the intensity and duration of the treatment. Plants gradually exposed to increasing (or decreasing) temperatures have the ability to acquire thermotolerance, adapting to withstand the stress (Correia *et al.*, 2011; Chaves *et al.*, 2011a; Correia *et al.*, 2012). Cork oak requires an annual mean temperature of 13–18°C (Gil and Varela, 2008). It is sensitive towards low temperatures, specially freezing or abrupt temperature drops (Aranda, *et al.*, 2005, Gil and Varela, 2008). However it has the ability to acclimate to drought (by investing on a deep root system) and high temperatures, characteristic of it's habitat during summer (Correia *et al.*, 2011; Correia *et al.*, 2012). In fact, under controlled conditions, and after being exposed to gradually increasing temperatures (starting in 25ºC and increasing 10ºC each 3 days) 8 months-old cork oak plants maintained the efficiency of the photosystem II up to 55ºC (showing best results until 45ºC) while well watered; however, after 3 days at this high temperature the tree started to show signs of severe stress. Electrolyte leakage increase between 35-45ºC, decreasing in higher temperatures, which also indicates an acclimation process (Correia *et al.*, 2011; Correia *et al.*, 2012).

In the recent decades, there has been an increasing interest in cork oak, with the widespread death among these trees. Some trees show sudden death, while others resist death and slowly decay (Pereira and Caldeira, 2011; Chaves *et al.*, 2011b; Correia *et al.*, 2011; Thomas

et al., 2002). It is thought that *Phytophthora cinnamomi* (fungus, Figure 6) is a main cause of cork oak sudden death, as it has been reported to other pine and oak trees (Ivors *et al.*, 2008; Thomas *et al.*, 2002). It infects the roots of the trees and makes it weaker, so that further biotic (insects, other microorganisms) and abiotic challenges, that the tree could otherwise withstand, could be fatal (Inácio *et al.*, 2011; Horta *et al.*, 2011, Thomas *et al..*, 2002; Ivors *et al.*, 2008).

Figure 6: Appearance of a non-inoculated Fraser fir seedling compared to those inoculated with *Pythium vexans* isolate or co-inoculated with *Phytophthora cinnamomi* (Ivors *et al.*, 2008)

Associated with climate changes and biotic stress, agro-forestall practices may also have influenced the decrease in cork oak population. The agricultural methods in *montados* may harm the root systems of the trees that may lead to injure and death of the plants when associated with drought and *P. cinnamomi* infections,. (Pereira and Caldeira, 2011; Koskela *et al.*, 2007, Chaves *et al.*, 2011b, Schaffhauser *et al.*, 2011, Vogiatzakis and Careddu, 2003). Water loss (and damage by summer fires) is also enhanced by debarking. The method is harmful for the tree, as its trunk becomes exposed to high temperatures, with the removal of the protective bark a large amount of water is lost and the tree must invest resources in healing (Correia *et al.*, 1992).

3. Main goals and work outline

In the late decades there has been a large decrease in the cork oak population, due to diseases, agricultural politics and climatic changes. Due to its economical and ecological importance, there has been an increased interest in understanding, recovering and maintaining

these trees (Koskela *et al.*, 2007, Chaves *et al.*, 2011b, Schaffhauser *et al.*, 2011, Gil and Varela, 2008).

In 2009 a group of Portuguese labs joined forces in the COEC (http://coec.fc.ul.pt/), in order to build and characterize cork oak EST libraries for several situations: development (e.g. cambial differentiation, flower development, fruit and embryo development), biotic (*Phytophthora cinnamomi* infection) and abiotic (drought, salt, heat, cold and oxidative) stress responses, cork production and quality. Several assays were performed, and different biological material collected (depending on the library) for the several situations. Total RNA was extracted and sequenced by 454-pyrosequencing, at BIOCANT (Cantanhede, Portugal). Normalized and non-normalized libraries of transcripts were prepared with the sequencing data and set in an online platform (CorkOak DB, http://corkoakdb.org/). Two pipelines were also developed, one for detecting SNPs (Single Nucleotide Polymorphisms) and another, developed within the SuberStress project, to analyze gene transcription levels from the non-normalized libraries, in order to find key regulatory genes in networks of stress response.

The goal of the SuberStress project is to understand which genes may be involved in the cross-talk between different imposed stresses by the analysis of expression variations in all the tested biotic and abiotic conditions. Starting from EST libraries obtained within the COEC, a bioinformatics approach would select genes showing to be differentially expressed in stress *vs*. control conditions, which could be common to several stresses. For functional validation of the selected genes, all the biotic and abiotic stress assays are being repeated, in order to collect biological samples (leaves and/or roots), as well as physiological data. The physiological data will be used to access the response of the plants to stress, whereas the biological material will be used to validate the genes selected *in silico* by RT-PCR.

The SuberStress project includes several partners from different Institutes, and each one of them is in charge of a different stress condition. The work described in the present report is integrated within the first tasks of the SuberStress project, which had two major goals, mainly focusing on temperature stress (heat and cold):

- Extract RNA samples from leaves previously exposed to heat and cold stress assays in order to prepare the non-normalized EST libraries through 454 pyrosequencing.

- Perform a new heat stress assay using cork oak plants, in order to collect RNA samples for candidate gene validation.

- Determine the expression pattern of several cork oak candidate genes in response to in heat and cold stress.

Chapter 2. Prepare and analyze non-normalized EST libraries for heat and cold stress

2.1. Summary

SuberStress project follows the work started in the COEC and aims to characterize putative regulatory genes in cork oak response to stress. This work in particular aims to gain a better insight of regulatory genes of thermal stress response (heat and cold). Total RNA was extracted and purified from leaf samples collected during heat and cold stress assays, previously performed within the projects of the COEC. The mRNA was sequenced by 454-pyrosequencing at BIOCANT. Sequencing data, along with data previously obtained from non-normalized libraries for the drought and biotic stress, were analyzed in order to search for genes with relevant expression changes in stress response in cork oak. The bioinformatic analysis of the EST libraries was performed by other members of the team participating in the SuberStress project. From this analysis, a group of 27genes was selected.

2.2. Plant material and Methods

Heat and cold stress assays had been previously performed, using potted 28 months old cork oak half-sibling plants. They were germinated from seeds collected at Tapada da Ajuda (38º43'6"N latitude, 9º11'27"W longitude and 101m altitude). The plants were pruned to stimulate leaf spouting and stress assays were applied around 6 weeks after. Plants were maintained into walk-in Fitoclima^{TM} chambers (Aralab) with 12h photoperiod, around 600 $mmol.m^{-2}.s^{-1}$ irradiance, 50%RH and around 380 $mL.L^{-1}.CO_2$, at 25/20ºC (day/night). Plants were watered regularly to field capacity. Young fully expanded leaves were collected and used for isolation of total RNA.

For heat stress assay, plants were separated in two groups: one group was subjected to a shock temperature of 42ºC for 10h and the other was subjected to progressively higher temperatures along the assay (17 days), with a one degree increase every day. Leaves from 6-8 plants were collected after 2 hours of illumination (day time) and immediately frozen in liquid N_2 and stored at -80ºC before RNA purification. They were collected at day 0 (2h, 5h and 10h after day photoperiod start, for 25ºC and the 42ºC shock temperature), and then at days 8, 13 and 17 (32/23ºC, 38/26ºC and 42/28ºC), respectively (Figure 7).

For cold stress assay, plants were separated in two groups: one was subjected to a shock temperature of 5ºC (chilling) for 10h and the other was subjected to progressively lower temperatures along the experiment, with a one degree decrease every day (up to 3ºC/-2ºC, day/night). For the acclimation group, at the temperature 15/10ºC, some plants were transferred to a shock temperature o of -2ºC (freezing). Leaves from 6-8 plants were collected after 2 hours of illumination (day time) and immediately frozen in liquid N_2 and stored at -80ºC before RNA purification. They were collected after 2h, 5h and 10h for the chilling and freezing shocks, and at 15/10ºC, 10/5ºC, 5/0ºC and 3/-2ºC for the acclimation treatment (Figure 7).

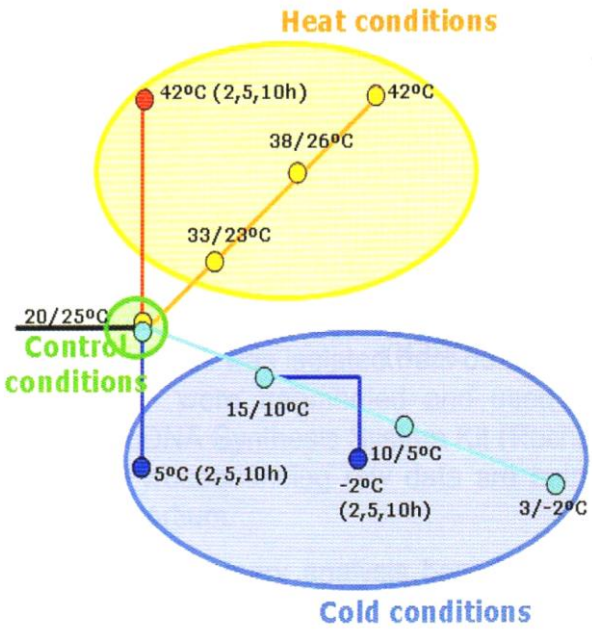


Figure 7: Representative schemes of the heat and cold stress assays (Chaves *et al.*, 2011a).

2.3. RNA extraction and quantification

Several time-point samples were taken, but only 6 were selected for sequencing: samples 42ºC (5h) for the heat shock and 42ºC for the heat acclimation, sample 10ºC for the cold acclimation, sample of 5ºC (5h) for the chilling shock and the control samples (25ºC, 2h) for each assay.

The leaf samples were ground into a fine powder, with mortar and pestle in liquid N_2. Total RNA was extracted from 100mg of frozen leaf powder with Spectrum Plant Total RNA kit™ (Sigma-Aldrich Co, LLC.) according to manufacturer's instructions. After extraction the samples were purified using RNeasy MinElute Cleanup kit™ (Qiagen), according to manufacturer's instructions, and treated for DNA contamination with Turbo DNA- free™ kit (Ambion, Life Technologies, USA). The RNA was quantified using Nanodrop™ UV-Vis Spectrophotometer (Thermo Scientific NanoDrop 2000, USA). The quality of the samples was assessed by the A260/A280 and A260/A230 absorbance ratios. RNA integrity was de-

termined by electrophoresis, applying 1-2µg of each RNA sample in 1% agarose gel with ethidium bromide staining. The RNA gel was visualized using the Image Analyzer Gel Doc™ XR+ (Biorad, USA). A total of 4 replicates were extracted and purified for each time-point. The purified RNA samples were sent to BIOCANT for sequencing. However RNA samples still had contaminants that were inhibitors to sequencing by 454-pyrosequencing, so, since the purification protocols of the first extractions did not eliminate the contaminants, we repeated the total RNA extractions, without further purification steps, for a total of 7 and 8 replicates (for the heat and cold time-points, respectively). The new samples were frozen and sent to BIOCANT, where they were purified using an optimized protocol (PVP_{10} Purification protocol, see Additional Information section) and sequenced.

2.4. Results and Discussion

2.4.1. RNA extractions and EST Sequencing

A first set of RNA samples was obtained following the protocol previously described (Figure 8). With this protocol we obtained 4 to 18ug of total RNA with A260/A280 ratios close to 2.0 (Table 1). However the A260/A230 ratios obtained were around 1.0, indicating possible contamination of polysaccharides, which may inhibit sequencing reactions. To circumvent this problem, BIOCANT suggested a cleanup protocol using PVP_{10} which, although effective, would require larger amounts of total RNA. In this way we performed another set of extractions for specific samples (Table 1). These were sent to BIOCANT (without any further cleanup) where the PVP_{10} purification protocol was applied with success. These samples were further used in 454-pyrosequencing reactions. The number and average size of raw reads obtained from the sequencing of each library are listed in Table 2.

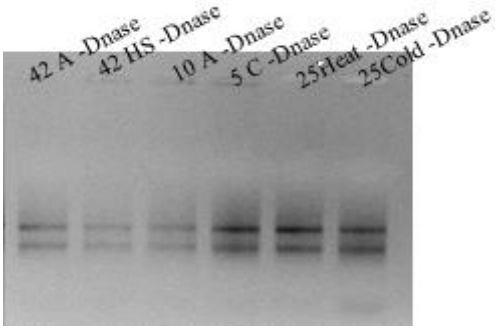


Figure 8: 1% agarose gel for the RNA samples (2µg) from the 1st extraction, after the DNase treatment. RNA samples from cold (CSA), heat shock (HSA) and heat acclimation (HAA) stress assays. 25HEAT/COLD: control samples; 5C, 42HS: cold and heat shock samples; 10A, 42A: cold and heat acclimation samples.

Table 1: Average RNA amounts and absorbance ratios. Data measured with Nanodrop ND-2000™.

Sample	1st extraction (after purification protocol and DNase)			2nd extraction (without purification protocol or DNase)		
	Total m_{RNA} (µg)	A260/A280	A260/A230	Total m_{RNA} (µg)	A260/A280	A260/A230
42A	7,85	1,95	0,81	1050,02	1,51	0,51
42HS	5,77	1,85	1,03	1003,06	1,55	0,51
10A	4,55	1,86	0,71	625,38	1,46	0,44
5C	6,06	1,91	0,64	557,28	1,44	0,45
25H	18,69	2,01	1,43	-	-	-
25C	12,03	1,90	1,15	-	-	-

Table 2: Number of reads from each EST library (heat and cold stresses).

	No. raw reads	Average length (bp)	Standard deviation
IC_42A	103928	236,7737	117,2699
IC_42HS	180347	242,0716	109,4480
IC_10A	211017	251,3714	117,4461
IC_5C	210185	257,5715	117,2699
IC_25H	97357	266,8884	115,3156
IC_25C	96530	249,3979	109,9979

2.4.2. Analysis of the non-normalized EST libraries

The data from the non-normalized EST libraries for abiotic and biotic stresses were processed by the bioinformatics team working in this project. *In silico* expression analysis was conducted by comparing each stress library with the corresponding control library. Sets of genes showing differential expression (>2-fold down- and upregulation) for each stress were intersected and a group of 27 candidate genes was selected for functional validation (Table 3). This selection was based on predicted gene function and levels of enrichment/depletion among different stresses.

Table 3: Candidate genes selected for functional validation. Predicted function was obtained by homology search using Blast2GO (Conesa and Götz, 2005).

Unigene ID	Given name	Homology	Predicted expression pattern
mlasm200_c6427	*QsUNK1*	uncharacterized protein	- Upregulated by heat acclimation, drought and infection in seeds
mlasm200_rep_c717	*QsNAC4*	nac domain protein nac4	- Upregulated by heat acclimation, drought and infection in seeds
mlasm200_rep_c98406	*QsAQP*	Aquaporin	- Upregulated by heat acclimation and infection in plants
mlasm200_rep_c3522	-	eid1-like f-box protein 3-like	- Upregulated by heat acclimation and drought
mlasm200_c12944	*QsEMB506*	ankyrin repeat domain-containing protein (EMB506)	- Upregulated by heat and drought
mlasm200_rep_c4654	*QsUNK2*	uncharacterized protein	- Upregulated by cold acclimation and drought

mlasm200_rep_c96982	-	protein (enolase)	- Upregulated by cold acclimation and infection in plants
mlasm200_rep_c93235	*QsDH*	Dehydrin	- Upregulated by cold acclimation and drought
mlasm200_rep_c9010	-	dehydration-responsive element binding protein 2	- Upregulated by cold and drought - Downregulated by infection
mlasm200_rep_c25840	*QsWRKY33A*	probable wrky transcription factor 33-like	- Upregulated by cold acclimation, drought and infection in plants - Downregulated by infection in seeds
mlasm200_rep_c337	*QsZFC3H*	zinc finger ccch domain-containing protein	- Upregulated by cold shock and infection in plants
mlasm200_rep_c1042	*QsWRKY33B*	probable wrky transcription factor 33-like	- Upregulated by cold acclimation, drought and infection
mlasm200_rep_c1777	-	ap2 erf domain-containing transcription factor	- Upregulated by cold shock and infection
mlasm200_c6039	*QsRAV*	ap2 domain-containing transcription factor (RAV)	- Upregulated by cold shock and infection
mlasm200_rep_c21427	*QsERF5*	ethylene-responsive transcription factor 5	- Upregulated by cold shock and infection in plants - Downregulated by drought
mlasm200_rep_c2884	*QsUBC2-17*	ubiquitin-conjugating enzyme e2-17 kda	- Upregulated by cold and infection in seeds
mlasm200_rep_c11855	*QsLTI6B*	low temperature and salt responsive protein lti6b	- Upregulated by cold acclimation and drought
mlasm200_rep_c8475	*QsPLIPA*	plastid-lipid-associated protein	- Upregulated by cold acclimation and drought
mlasm200_rep_c97597	*QsGluS*	glutamine synthetase	- Downregulated by heat and cold acclimation and by drought
mlasm200_c12471	-	zinc finger protein nutcracker-like	- Downregulated by cold, drought and infection in plants - Upregulated by infection in seeds
mlasm200_c2143	-	thioredoxin superfamily protein	- Downregulated by cold and drought - Upregulated by infection in seeds
mlasm200_rep_c2145	-	cyanate hydratase	- Downregulated by cold and drought - Upregulated by infection in seeds
mlasm200_rep_c3556	-	abc transporter family protein	- Downregulated by heat and drought - Upregulated by infection in seeds
mlasm200_rep_c5380	-	thioredoxin h2	- Downregulated by heat acclimation - Upregulated by infection
mlasm200_c952	-	probable anion transporter chloroplastic-like	- Downregulated by heat acclimation and infection

mlasm200_rep_c50446	-	s-adenosylmethionine decarboxylase	- Downregulation by heat - Upregulation by infection in plants and drought
mlasm200_rep_c825	*QsO3FAD*	omega-3 fatty acid desaturase	- Downregulated by heat acclimation - Upregulated by infection in seeds and drought

In search for putative housekeeping genes (HKGs) we considered the results obtained by previous studies for cork oak by Marum *et al.* (2012) and Chaves *et al.* (2011b) which suggested that *glyceraldehyde-3-phophate dehydrogenase (GAPDH), Clathrin adaptor complexes medium subunit family* (*CACs), Actin (Act), ß-Tubulin (ß-Tub)* and *HMG-CoA synthase (HMG)* as good HKGs.

In addition, other candidate HKGs were selected from *in silico* analysis using the non-normalized libraries for abiotic and biotic stress. Based on predicted gene expression, a group of genes that showing lowest variance between libraries was identified and from these we designed primers for one of the most promising, *Proteasome small subunit β* (*QsProtSß*) (Table 4).

Table 4: Putative *Q. suber* housekeeping genes, for thermal stress.

Unigene ID	Given name	Homology	Source
ID 6728500	*QsCACs*	Clathrin adaptor complexes medium subunit family	Marum *et al.*, 2012
EU 697020	*QsAct*	Actin	
ID 6744996	*QsGAPDH*	Glyceraldehyde-3P dehydrogenase	
-	*PrdBTub*	B-tubulin	Marum *et al.*, 2012 Primers designed for *Prunus dulcis* β-tubulin unigene
-	*QsHMG*	HMG-CoA synthetase	Chaves *et al.*, 2011b
mlasm200_rep_c435	*QsProtSb*	Proteasome small subunit β	One of the unigenes that showed lower variability among the stress libraries

Chapter 3. Heat stress assay applied to 4 years old *Quercus suber* plants

3.1. Summary

To perform a functional validation of the candidate genes selected based on the previously described work we repeated the heat stress assay to obtain new RNA samples.

A group of 60 potted 4 years-old cork oak half-sibling plants were used in the assay, consisting in acclimation and non-acclimation situations. To better understand cork oak response to heat stress we recorded physiological data along the assay with the collecting of leaves. This assay is a replicate of the one used to sampling for the EST libraries.

3.2. Plant material and Methods

Four years old potted cork oak half-sibling plants (see section 2.2.) were transferred to a walk-in Fitoclima chamber (Aralab). Light intensity at plant level was about 450µmol.m^{-2}.s^{-1}). Dormancy was induced for 14 days (plants were kept at 25º/20ºC (day/night) for 4 days, then temperature was decreased to 18º/13ºC for another 4 days and afterwards to 10º/5ºC for 6 days). After this time, the plants were pruned and temperature was increased to stimulate vegetative growth (7days at 18º/13ºC day/night and afterwards 25º/20ºC until the assay started). Plants were kept during 1 month at 25ºC/20ºC (day/night) temperature, 12h/12h photoperiod, 380ppm CO_2 concentration and 45% RH (control conditions). They were watered daily, at the beginning of the day photoperiod, to guarantee that soil water content was not a limiting factor. Pots were randomly rotated, but not moved to minimise position effects. For heat stress treatment plants were separated in two groups: one group was subjected to a shock temperature of 42ºC for 5h and the other was subjected to progressively higher temperatures along the experiment (18 days), with a one degree increase every day.

Leaf gas exchange was monitored along the experiment with a portable infrared gas analyzer equipped with a fluorometer (LI-6400-40, LI-COR Inc., Lincoln, USA). We have measured net CO_2 assimilation (An, µmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (gs, mol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (Ci, ppm). Simultaneous measurements of chlorophyll *a* (Chl *a*) fluorescence made possible to estimate the operating efficiency of the photosystem II (Φ_{PSII}), which represents the efficiency of the photochemistry.

Measurements were done 2h after photoperiod starts, in fully expanded leaves from the middle/lower insertion position of the shoot from plants grown in different pots (6-9 plants/timepoint). Groups of 3 leaves from 4 plants were for each timepoint and immediately frozen in liquid N_2 and stored at -80ºC before RNA purification (Figure 9).

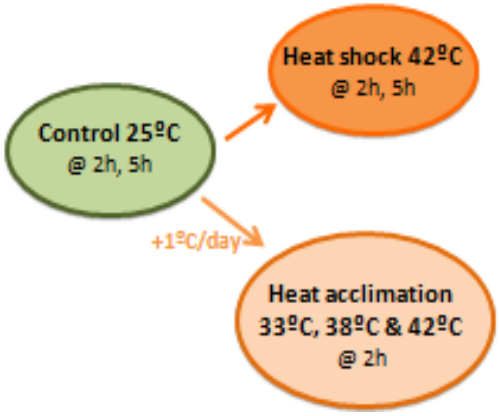


Figure 9: Heat stress assay. Plants were subjected to acclimation (gradual temperature increase) and non-acclimation (shock temperature) to heat stress. Leaves (pool of 3leaves from 4plants) were collected 2h after starting day photoperiod for control, shock and acclimation samples; also collected after 5h for the control and shock situations. Physiological data were registered with a portable infrared gas analyzer equipped with a fluorometer (LI-6400-40, LI-COR Inc., Lincoln, USA).

Measurements and plant material collection were done at day 0 (2h and 5h after day photoperiod start, for 25ºC and the 42ºC shock temperature), and then on days 8 (33ºC), 13 (38ºC) and 17 (42ºC). Measurements were done without controlling block temperature and using a light intensity set at 450 µmol photons m^{-2} s^{-1} (10% blue light) with a CO_2 concentration maintained at 400 ppm and an air flow rate of 500 µmol s^{-1}.

3.3. Results and Discussion

From the measurements taken for the several time-points, graphics were drawn for Net CO_2 assimilation (An), stomatal conductance to water vapor (gs) and internal CO_2 concentration (Ci) and Operating efficiency of the photosystem II (Φ_{PSII}) (Figures 10 and 11). The standard error was calculated for each parameter.

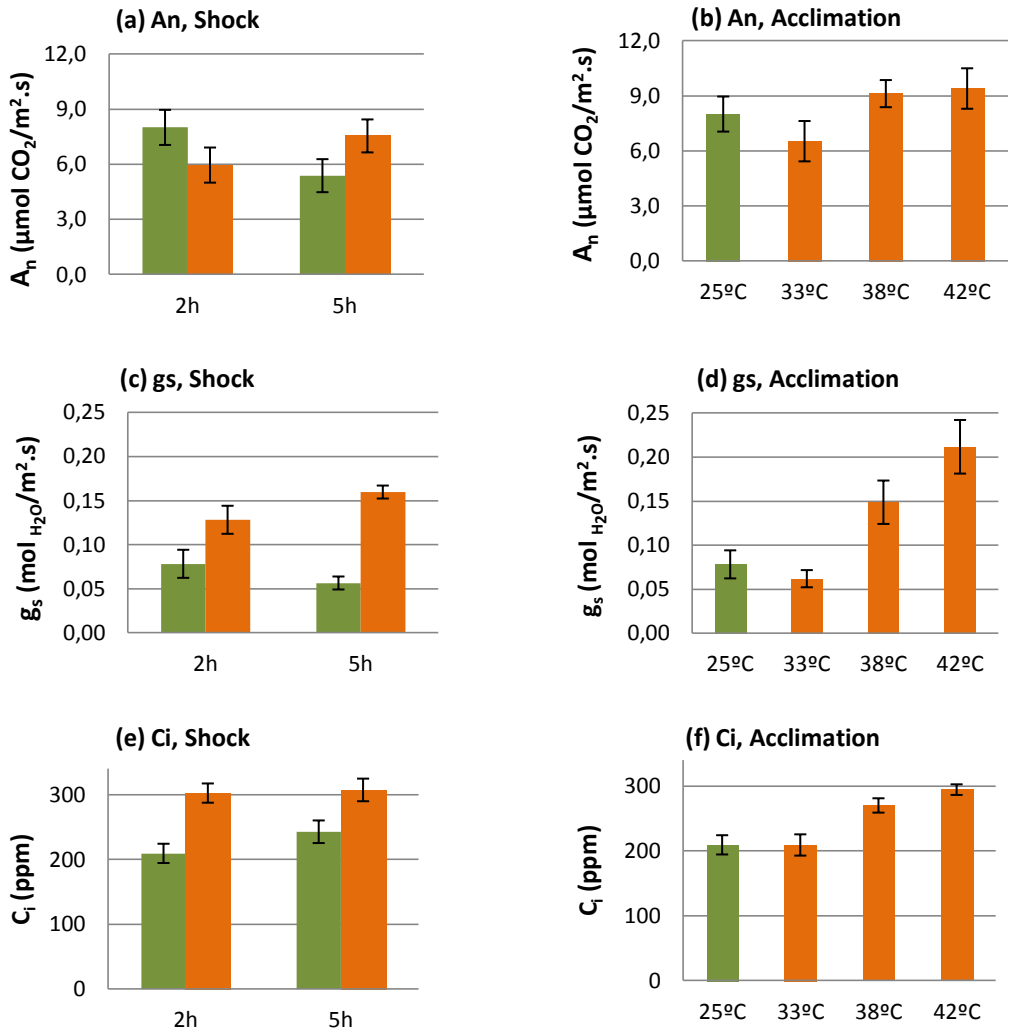


Figure 10: Net CO_2 assimilation [An, (a) and (b)], stomatal conductance to water vapor [gs, (c) and (d)] and internal CO_2 concentration [Ci, (e) and (f)] measured in both treatments during heat shock and heat acclimation. Values are means ± SE (n=6-9). Represented in green are the control samples (25ºC) and in orange the heat stress samples.

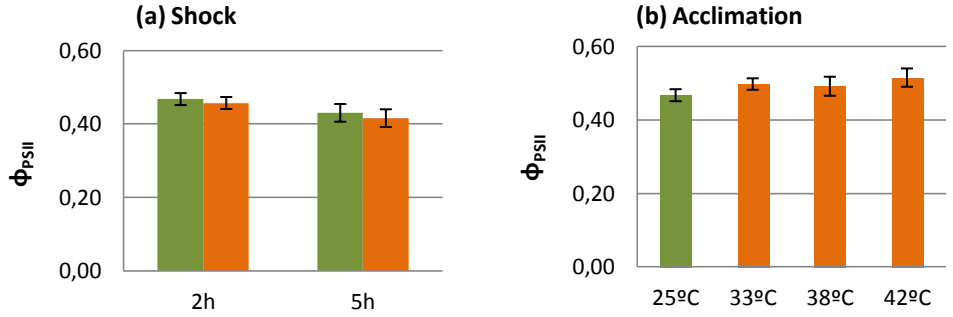


Figure 11: Operating efficiency of the photosystem II (Φ_{PSII}) measured in both treatments: heat shock (a), and heat acclimation (b). Values are means ± SE (n=6-9). Represented in green are the control samples (25ºC) and in orange the stress samples.

Considering both the heat shock and acclimation, there is a clear increase in the stomatal conductance and in the internal CO_2 concentration with the increasing temperature (Figure 8). The fact that plants were always kept in well-watered conditions may explain this response, since it would allow them to increase transpiration and reduce leaf temperature, opening the stomata (Hamerlynck, *et al.,* 1995).

Our results suggest that under the tested conditions, heat stress (shock and acclimation) has no major effect on photosynthesis, as Φ_{PSII} were maintained during heat stress (Figure 9). That may be associated to the fact that cork oak trees must be adapted to resist to warmer environment, so that 42ºC may not be a stronger challenge, or the exposure time was not long enough to show damage (Kadir and Von Weihe, 2007; Chaves *et al.*, 2011a). In the literature we can find reference to cork oak PSII being more sensitive to abrupt drops in temperature than abrupt increases (Aranda *et al.*, 2005, Chaves *et al.*, 2011b). Another study also showed that cork oak plants keep their photosynthetic efficiency exposed to temperatures up to 55ºC (Correia *et al.*, 2011; Correia *et al.*, 2012). These authors observed that plants started to decrease their performance above 45ºC but endured 3day exposure to 55ºC until clearly starting to show signs of severe stress and senescence in the leaves. Previous studies also showed that acclimation to heat stress leads to an increased stability of the photosystem II apparatus, also leading to an increase of the critical temperature (*i.e.* the temperature above which the photosynthetic apparatus is irreversibly damaged) (Kadir and Von Weihe, 2007). Considering this and that it our assay was conducted in a well-watered environment, the maintenance of Φ_{PSII} is to be expected for acclimation.

To confirm this data, a new assay will be performed, and we aim to measure additional parameters to assess plants response (for example: calculate and control RWC, determine RuBisCO activity, electrolyte leakage and quantify chlorophyll a/b content). Considering the natural cork oak genetic variability, this new assay should have a larger number of measurements (at least 3 leaves *per* plant and at least 8-10 plants *per* timepoint).

Chapter 4. Expression analysis of putative genes of interest in response to stress

4.1 Summary

In Chapter 2 we have reported the identification of several candidate genes which could play a role in cork oak response to stress. In order to study the expression pattern of the candidate genes in response to thermal stress, we optimized the RNA extraction protocol for cork oak leaves. Primers were designed for the candidate genes and for the candidate house-keeping genes and gene expression analysis was conducted using semi-quantitative RT-PCR. In this Chapter we report the expression pattern of 16 of the 27 candidate genes selected, in response to heat stress (using the samples collected in the heat stress assay described in Chapter 3) and cold stress (using RNA samples from the cold stress assay described in Chapter 2).

4.2. RNA extractions

4.2.1. Material and Methods

RNA extraction turned out to be a limiting step in this work, and we had to try several extraction protocols in combination with different RNA purification strategies (to remove sample contaminants, such as polysaccharides) to guarantee maximum yields and high purity. All the protocols tested are mentioned in Table 5 and described in Additional Information section. All the extractions were repeated to have technical replicates. RNA extraction was performed using, a pool of leaves from 3 different individuals for each time-point. For the cold stress we used the leaf samples collected for the non-normalized libraries (described in Chapter 2; samples control, 5ºC chilling shock and 10ºC acclimation).

Total RNA samples were quantified using Nanodrop UV-Vis Spectrophotometer (Thermo Scientific NanoDrop™ 2000, USA). The quality of the samples was assessed by the A260/A280 and A260/A230 absorbance ratios. RNA integrity was determined by electrophoresis, applying 250ng of each RNA sample in 1% agarose gel with ethidium bromide staining. The gel was visualized using the Image Analyzer Gel Doc™ XR+ (Biorad, USA). All the good quality samples, selected to cDNA synthesis for the RT-PCRs, were treated with DNAse (Ambion's Turbo-DNA free kit™, according to manufactures' instructions).

Table 5: List of total RNA extraction and purification protocols used. The ones highlighted showed the best results: CTAB for heat shock/control samples and Hot Borate for heat acclimation and cold samples (followed by ethanol/NaOAc precipitation).

Extraction Protocols	Purification Protocols
Spectrum Plant Total RNA kit™ (Sigma)	**Turbo DNA-free kit™ (Ambion)**
Spectrum Plant Total RNA kit™ (Sigma) adapted by Brunner *et al.*.	RNeasy MinElute Cleanup kit™ (Qiagen)
RNeasy Plant Mini kit™ (Qiagen)	Total RNA Purification with PVP10
RNeasy Plant Mini kit™ (Qiagen) adapted by Brunner *et al.*	Total RNA Purification with PVP10 (adapted without columns' steps)
RNeasy Plant Mini kit™ (Qiagen) adapted by Gehrig *et al.*	**Ethanol/NaOAc Precipitation Protocol**
Direct-zol RNA MiniPrep kit™ (Zymo)	KOAc Precipitation Protocol
Plant & Fungi Total RNA Purification kit™ (Norgen)	RNeasy Mini kit™ (Qiagen)
CTAB Protocol adapted for eppendorf (Reid *et al.* 2006).	
Hot Borate Protocol (Wan & Wilkins, 1994)	

4.2.2. Results and Discussion

Some plant tissue samples can be difficult to extract, especially tissues samples collected in stress assays, due to the presence and accumulation of polysaccharides, polyphenols and other molecules that help to cope to stress (Reid *et al.*, 2006, Vasanthaiah *et al.*, 2008). As discussed in Chapter 2, the protocol used to extract RNA for 454 pyrosequencing required additional steps of purification, which would result in a decrease in RNA yield. To circumvent this problem we decided to invest in the optimization of a RNA extraction method for the samples collected during heat and cold stress. In the initial stage we used only the heat stress, specially the heat shock and control, samples. The samples from the acclimation to heat stress were the more problematic ones, since most extraction protocols generated viscous supernatants, making it very difficult to avoid carry over of debris to the next steps in the protocols. The cold stress samples were also difficult to extract, resulting in low concentration and quality.

From all the protocols tested, the CTAB (Reid *et al.*, 2006) and the Hot Borate (Wan and Wilkins, 1994) protocols (followed by ethanol/NaOAc precipitation) showed the best results. The other protocols produced low yield of low quality RNA (0,5-4,0ng with absorbance ratios A260/A280 around 1,5 and A260/A230 below 1,0; data not shown). The CTAB protocol (Reid *et al.*, 2006) produced good RNA quality and yield for heat control and shock samples. However this procedure did not show such promising results for the heat acclimation and cold stress samples (Table 6), resulting in low yield and quality.

For the cold stress and the heat acclimation samples the best RNA yield and quality was obtained with a combination of the hot borate extraction (Wan and Wilkins, 1994), followed by sodium acetate/ethanol precipitation protocols. Still, for the cold stress samples control and chilling shock we had to perform a second sodium acetate/ethanol precipitation to increase yield and purity (Figure 12). Even so, these samples showed a lower quality RNA, when comparing to the heat and cold acclimation samples (Table 6).

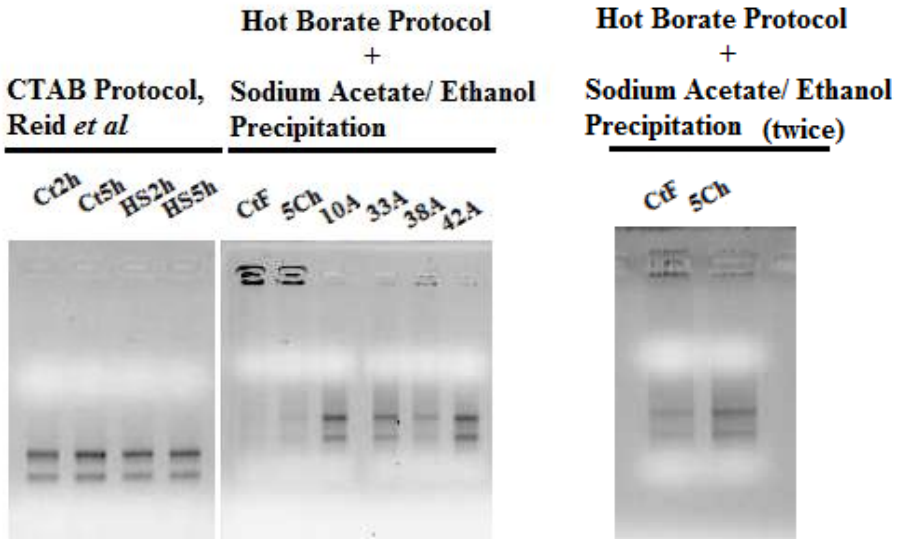


Figure 12: 1% agarose gel stained with ethidium bromide. Selected RNA samples (250ng) were extracted with the CTAB and hot borate protocols. RNA samples from cold (CSA), heat shock (HSA) and heat acclimation (HAA) stress assays. CtF, Ct2h, Ct5h: control samples; 5Ch, HS2h, HS5h: cold and heat shock samples; 10A, 33A, 38A, 42A: cold and heat acclimation samples. For CtF and 5Ch, a second purification step was necessary and the resulting RNA samples are shown in the rightmost gel.

Table 6: Average RNA amounts and absorbance ratios for the RNA samples extracted with CTAB (Reid *et al.*, 2006) and Hot Borate (Wan and Wilkins, 1994) Protocols. Data obtain with Nanodrop ND-2000.

Heat Shock Assay Samples	Sample	RNA amount (µg)	A260/A280	A260/A230
CTAB + DNase	Ct2h	7,6	2,06	1,89
	Ct5h	6,7	2,08	1,98
	HS2h	8,2	2,05	1,99
	HS5h	6,5	2,04	1,87
Cold Stress Assay Samples	**Sample**	**RNA amount (µg)**	**A260/A280**	**A260/A230**
CTAB	CtF	1,3	1,87	0,87
	5Ch	1,7	1,47	0,53
	10A	3,9	2,11	1,96
Hot Borate + EtOH/NaOAC + DNase	CtF	3,8	1,94	1,67
	5Ch	7,2	1,96	1,81
	10A	13,8	2,08	2,02
Heat Acclimation Assay Samples	**Sample**	**RNA amount (µg)**	**A260/A280**	**A260/A230**
CTAB	33A	0,2	2,26	0,91
	38A	0,6	1,60	0,61
	42A	0,2	1,91	0,95
Hot Borate + EtOH/NaOAC + DNase	33A	10,1	2,03	2,04
	38A	6,7	2,04	2,10
	42A	11,9	2,02	2,27

4.3. Gene expression studies

4.3.1. Materials and Methods

Primers for PCR amplification were designed for some of the genes in Tables 3 and 4, using primer3 online tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). cDNA was synthesized with Roche's High Fidelity cDNA synthesis kit™, according to manufactures' instructions and using Oligo-dT_{18} primers, starting with 500ng of each RNA samples (see section 4.2). cDNA samples (2 µL) were used as template for PCR amplification with 1x GoTaq reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.3µM of each primer, 3%(v/v) DMSO and 1U GoTaq® DNA Polymerase (Promega), in 20µL total volume. PCR was performed as follows: 5min incubation at 95ºC, followed by 22-36 cycles of 30sec at 95ºC, 30-40sec at corresponding annealing temperature (Table 7) and 40sec at 72ºC and a final extension step for 5min at 72ºC. The housekeeping genes selected were *QsActin* for cold and heat shock stresses and *QsProtSß* for heat acclimation stress. Technical PCR replicates were performed for each gene at least once. Total reaction volumes were analyzed by electrophoresis in 2% agarose gels with ethidium bromide staining, using GeneRuler DNA ladder mix™ as marker (Thermo Scientific). Images were captured using the GelDoc™ XR+ System (BioRad, USA) in standard conditions to all the gels (same amplification, focus, exposure time and applying the samples in gels with same size by the same order).

The PCR amplicons generated by all the specific primer pairs were sequenced to confirm specificity. To do this, a PCR for all the selected primer combinations was performed, in 50µl total volume, using a pool of stress and control cDNAs. In some cases, genomic DNA was used instead of cDNA. Although for most genes it was not necessary (they produced a single defined band when applied in agarose gel stained with ethidium bromide), for the *QsLTi6B, QsWrky33B* and *QsUnk1* genes the bands had a purification step before sequencing. To purify, the amplification reactions were applied in a 2% agarose gel stained with ethidium bromide. The bands corresponding to each of the genes were cut, purified with High pure PCR product purification kit™ (Roche) and the product was used as DNA template to new reactions, as described above. Amplification reactions were sent to Beckman-Coulter Genomics® (UK) to sequence. The sequenced amplicons were then aligned with the original transcript sequences using MultAlin online tool (http://multalin.toulouse.inra.fr/multalin/).

Table 7: Primers for the putative genes of interest and putative housekeeping genes. The optimized PCR conditions for each primer are also listed.

	Primer name	Primer sequence (bp)	Product length (bp)	T_{an} (ºC)	Cycles (CSA)	Cycles (HAS)	Cycles (HAA)
Genes of Interest	QsAQP_Fw	GACAGCTTCCTTGCCAAAAG	320	58,0	32	30	30
	QsAQP_Rv	GGCACATCAGCTCAGACAGA					
	QsEMB506_Fw	CTGCAGCACCAATTTCTTGA	406	58,0	32	28	30
	QsEMB506_Rv	ATGAAGTGGGCTCCATTTTG					
	QsDH_Fw	GGGATTCACAATCTGCTTGC	281	56,0	32	28	28
	QSDH_Rv	TCAAACCCACCAGTTCCATT					
	QsZFC3H_FW	ATTGGTTGGGTAGCTTGCAC	313	56,0	30	30	30
	QsZFC3H_Rv	CTTGCAGCTCCCTGGTAGTC					
	QsRAV_Fw	ATCGCTTGGAGTCACAGCTT	396	60,0	32	30	30
	QsRAV_Rv	GGTGCCCAGATTTACGAGAA					
	QsERF5_Fw	CTCAAGGGTATGGCTTGGAA	310	58,0	30	30	30
	QsERF5_Rv	ACTTGACGGCGTTAATGGAC					
	QsUBC2-17_Fw	ATGATCCTTTGGTGCCTGAG	294	58,0	25	28	28
	QsUBC2-17_Rv	TGCCACCAAGTTCCACATAA					
	QsGluS_Fw	TCTGTGTCTCGACCAACTCG	370	60,0	25	24	22
	QsGluS_Rv	AATTCCAAGTTGGCCCTTCT					
	QsO3FAD_Fw	ACAGTGTGGTGGGGCATATT	401	56,0	28	28	28
	QsO3FAD_Rv	CCAATAGGGAACGCCATAGA					
	QsUNK1_Fw	GCCACCTTGGTTGTGAAAGG	143	57,0	34	34	34
	QsUNK1_Rv	GTGGCTCATCTTCATCCAGG					
	QsNAC4_Fw	TCGCTTATTAGAGCACTCACG	108	58,0	34	30	30
	QsNAC4_Fw	ACACTTGTCGTTGGCTTCTGA					
	QsUNK2_Fw	CTTCTCCTTCGGTTGCAGAG	148	58,0	30	30	30
	QsUNK2_Rv	GCAGCGGACTCTTTCTCTTC					
	QsWRKY33A_Fw	GGAGTGGTAGTAGTAGTCCC	114	57,0	32	32	34
	QsWRKY33A_Rv	GCACAACAGGGATGAGCATG					
	QsWRKY33B_Fw	AGCCCCAGTAGTTGGAGATG	144	56,0	34	32	32
	QsWRKY33B_Rv	CCTCCTTCATTGCCACTCTC					

	Primer name	Primer sequence (bp)	Product length (bp)	T_{an} (ºC)	Cycles (CSA)	Cycles (HAS)	Cycles (HAA)
	QsLTI6B_Fw	TCCTCAGGTTTGGTTGCGAG	139	58,0	30	28	28
	QsLTI6B_Rv	TTGCCGTTCACAGGTCACAC					
	QsPLIPA_Fw	CCAAGTTTGAAGTCCGAAGTC	131	58,0	25	28	28
	QsPLIPA_Rv	AAGGTCAATCTTTTGCCCCAG					
Housekeeping (reference) genes	QsFw_CACs	AAGAGCCACCATTCAAATCC	390	56,0	30	30	30
	QsRv_CACs	TGACATCAGGTCGAGCAAAG					
	QsFw_Act	CCTTCCCCATGCAATTCTTA	294	56,0	30	28	28
	QsRv_Act	TCAATGAGAGATGGCTGGAA					
	QsFw_GAPDH	GATGCGATGTGGACAATCAG	300	56,0	25	22	22
	QsRv_GAPDH	CCGTGTTCCAACTGTTGATG					
	PrdTub_Fw	ATTGAGCGACCCACCTACAC	500	56,0	30	30	30
	PrdTub_Rv	GTGGGTGGCTGGTAGTTGAT					
	QsHMG-CoA_Fw	TGATGTATTCAACGAGTTTATAGC	±200	60,0	30	30	30
	QsHMG-CoA_Rv	TCCATACCTGTGCTCCATTAG					
	QsProtSb_Fw	CCATTGAAGGAGAAGGTGGA	407	58,0	30	28	28
	QsProtSb_Rv	GCTTTTGCGTTCAATCAACA					

Relative quantification of the RT-PCR products was achieved by quantifying the bands from the agarose gels using ImageJ software (Image processing and analysis in Java software, http://rsbweb.nih.gov/ij/index.html). Band intensities were normalized to the corresponding housekeeping gene (*QsActin* for cold and heat shock stresses and *QsProtSß* for heat acclimation stress).

4.3.2. Results and Discussion

Before assesssing the expression of the candidate genes during cold and heat stress assays, we determine the expression pattern of several housekeeping genes to be used as control. After comparing the patterns of six putative housekeeping genes (Figure 13), and taking in consideration the RNA pattern (Figure 12), *QsActin* was selected for the cold and heat shock assays and *QsProtSb* for heat acclimation.

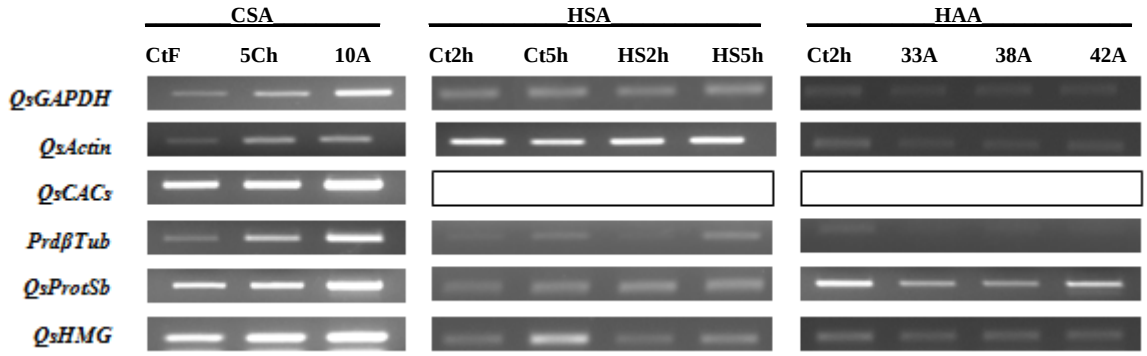


Figure 13: Representative expression patterns of six housekeeping genes during cold (CSA), heat shock (HSA) and heat acclimation (HAA) stress assays. CtF, Ct2h, Ct5h: control samples; 5Ch, HS2h, HS5h: cold and heat shock samples; 10A, 33A, 38A, 42A: cold and heat acclimation samples (2% agarose gels with ethidium bromide staining).

Sequencing results (Beckman) were aligned with the predicted amplicons obtained with the primers in table 7. We confirmed the sequences of most of the genes, including the HKGs *QsProtSb* and *QActin* (e.g. in Figure 14).

We could not confirm the sequences of the *QsDH, QsUnk1, QsWrky33A* and *QsNac4* genes. The sequencing results for these genes were of poor quality, according to the company's quality control standards. New RT-PCRs will be prepared, the corresponding bands will be purified and send to be sequenced.

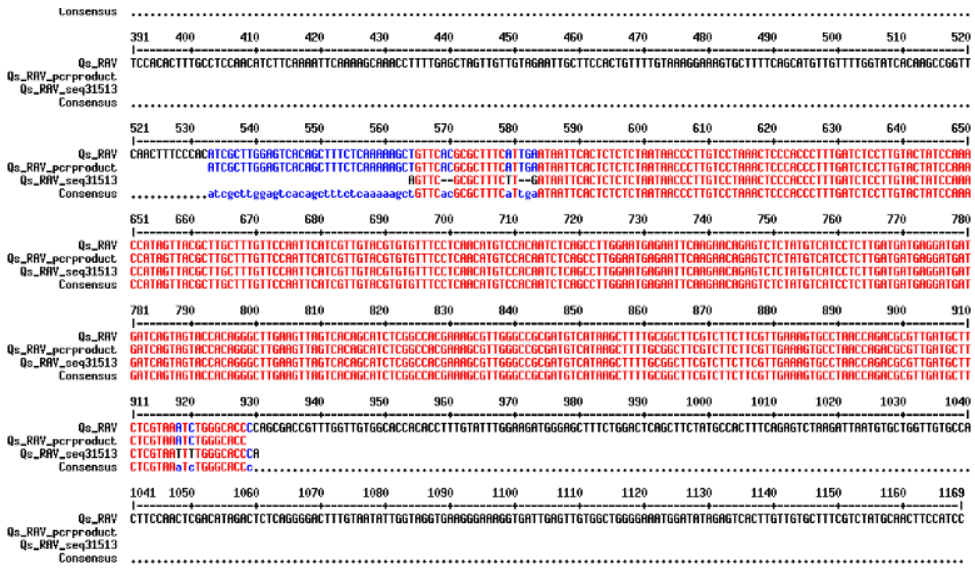


Figure 14: MultAlin alignment of the *QsRAV* unigene with the predicted amplicon and the sequencing result.

The expression patterns of the candidate genes during cold and heat stress assays (Figure 15) were compared with the predicted patterns *in silico* (Tables 3 and 8) and also with the patterns of the corresponding housekeeping genes.

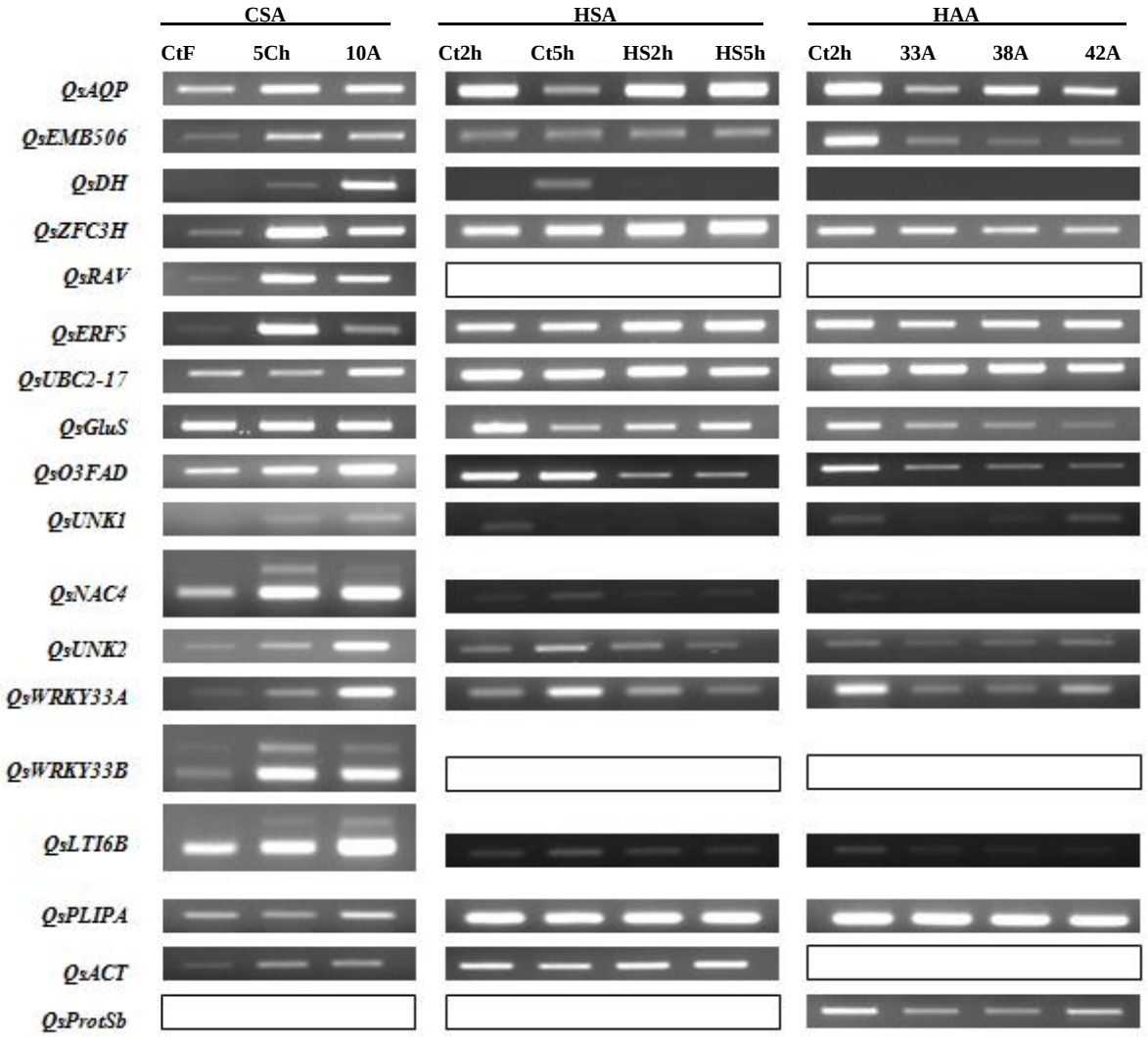
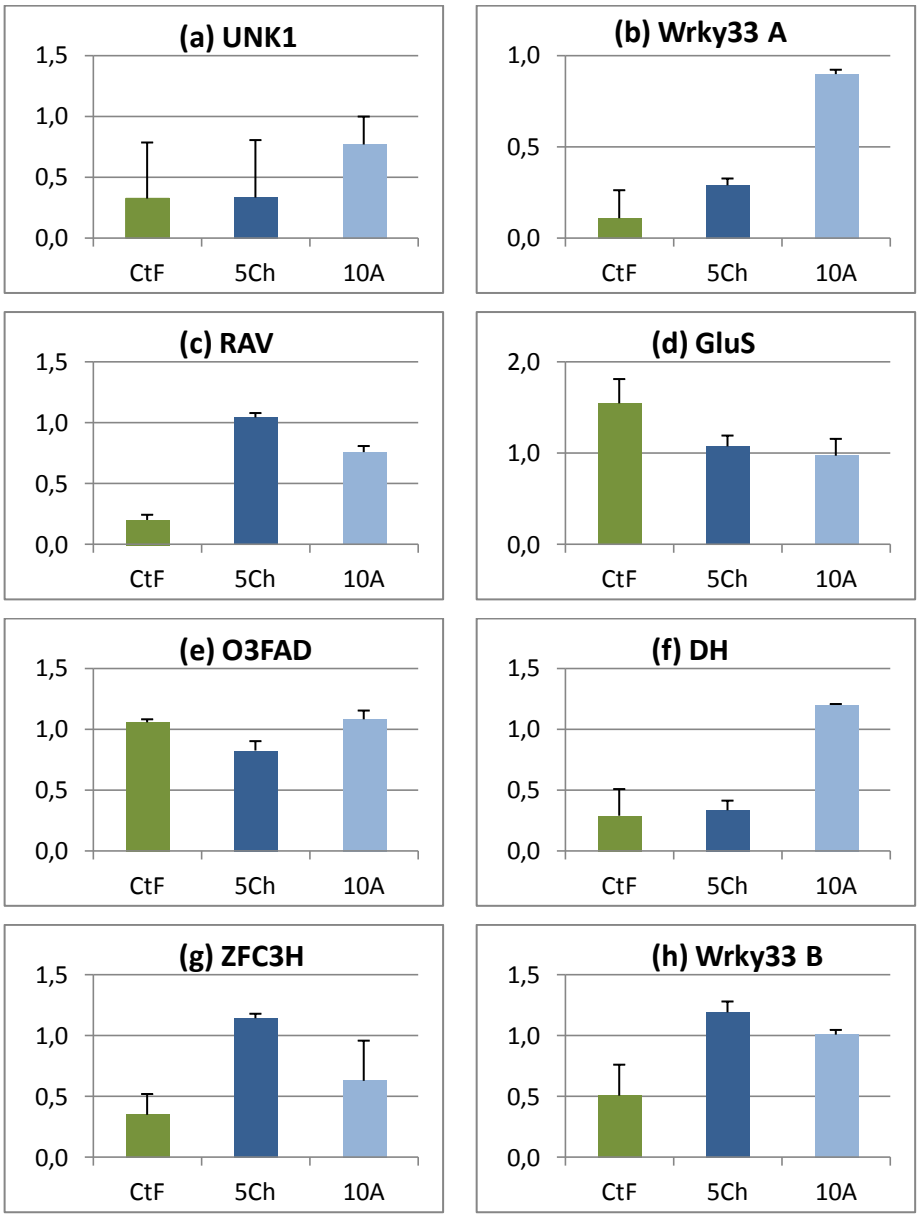


Figure 15: Representive expression patterns of the putative genes of interest in the CSA, HSA and HAA samples (see Table 7 and Figure 13; 2% agarose gels with ethidium bromide staining).

For the heat stress samples, *QsRAV* and *QsWrky33B* expression patterns will still need to be confirmed. One major bottleneck was to analyze expression patterns of the primer pairs designed for qPCR. The reduced size of the amplicon, associated with the, for some cases, apparent low expression, made difficult to conclude about the genes. That was the case, for example, of *QsWrky33B.*

QsNAC4, *QsLTi6B* and *QsWrky33A* seem to have an alternative splicing in the cold shock and acclimation samples (Figure 15). Interestingly these genes only show the alternative splicing for cold stress, not showing in control or heat.

To more easily compare each gene with the HKG, the intensity of the bands for each primer pair (for each treatment) was quantified using ImageJ software, and normalized to the corresponding HKGs' bands intensities (Figures 16, 17 and 18, for the cold, heat shock and heat acclimation treatments, respectively).



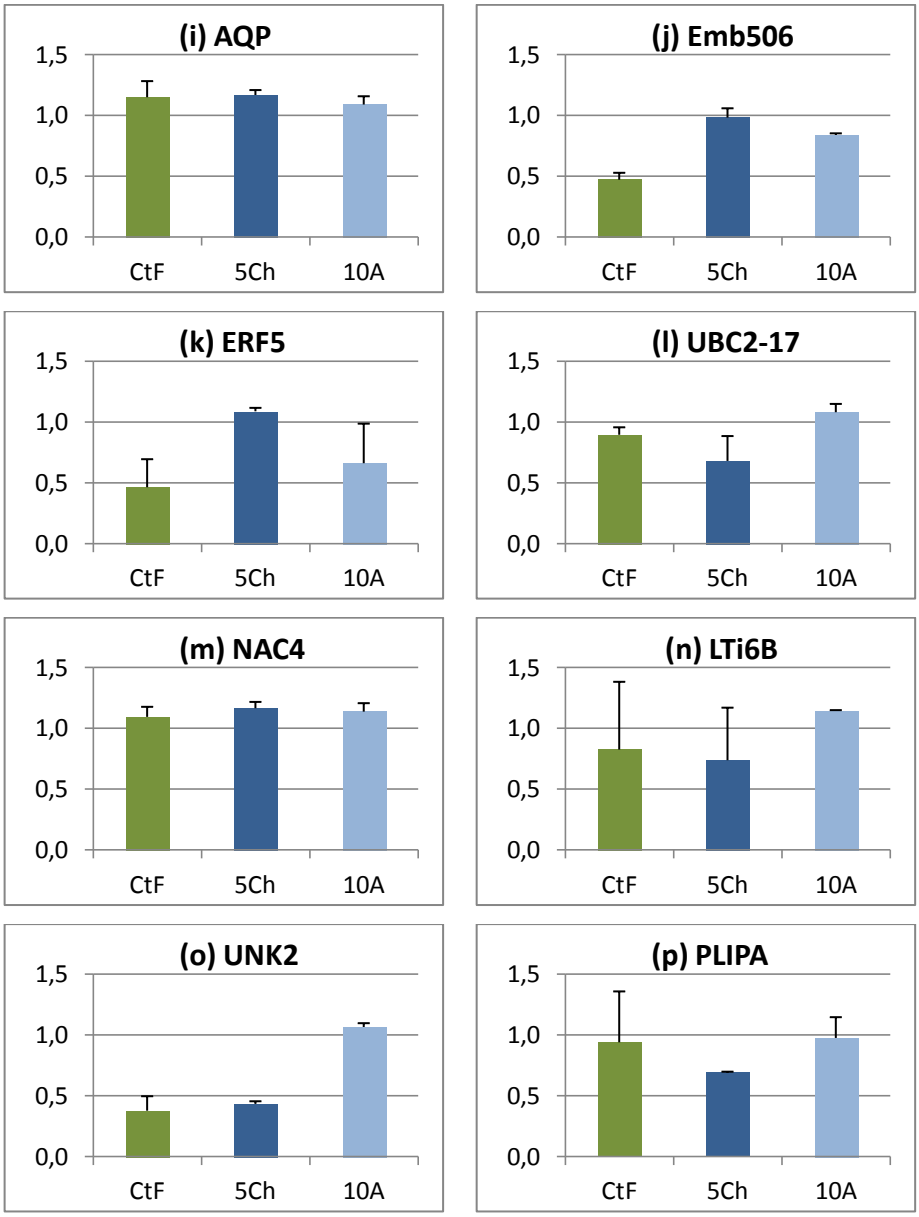
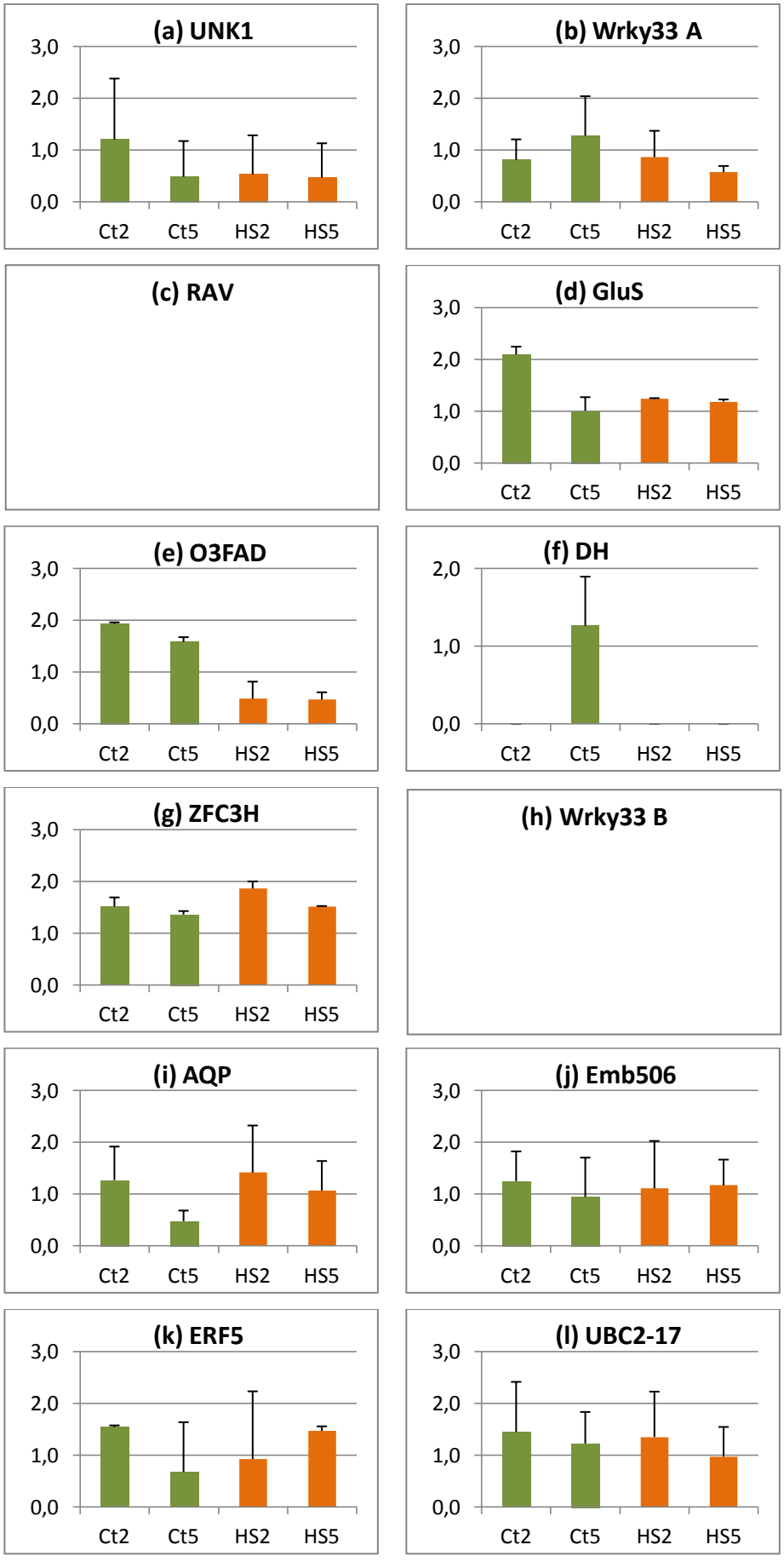


Figure 16 (a-p): Expression patterns of the putative genes of interest for the CSA samples (see Figures 13 and 15 and Table 7). Relative quantification of the RT-PCR using ImageJ software and normalizing all the genes to *QsActin*. Also represented the standard deviation (SD) for each timepoint.


(a) UNK1
(b) Wrky33 A
(c) RAV
(d) GluS
(e) O3FAD
(f) DH
(g) ZFC3H
(h) Wrky33 B
(i) AQP
(j) Emb506
(k) ERF5
(l) UBC2-17
3,0
2,0
1,0
0,0
Ct2
Ct5
HS2
HS5

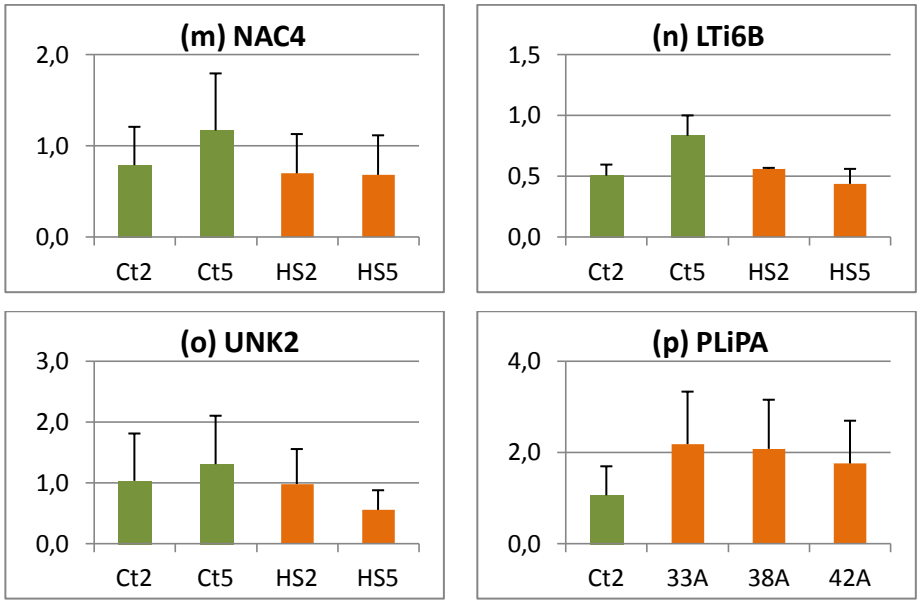
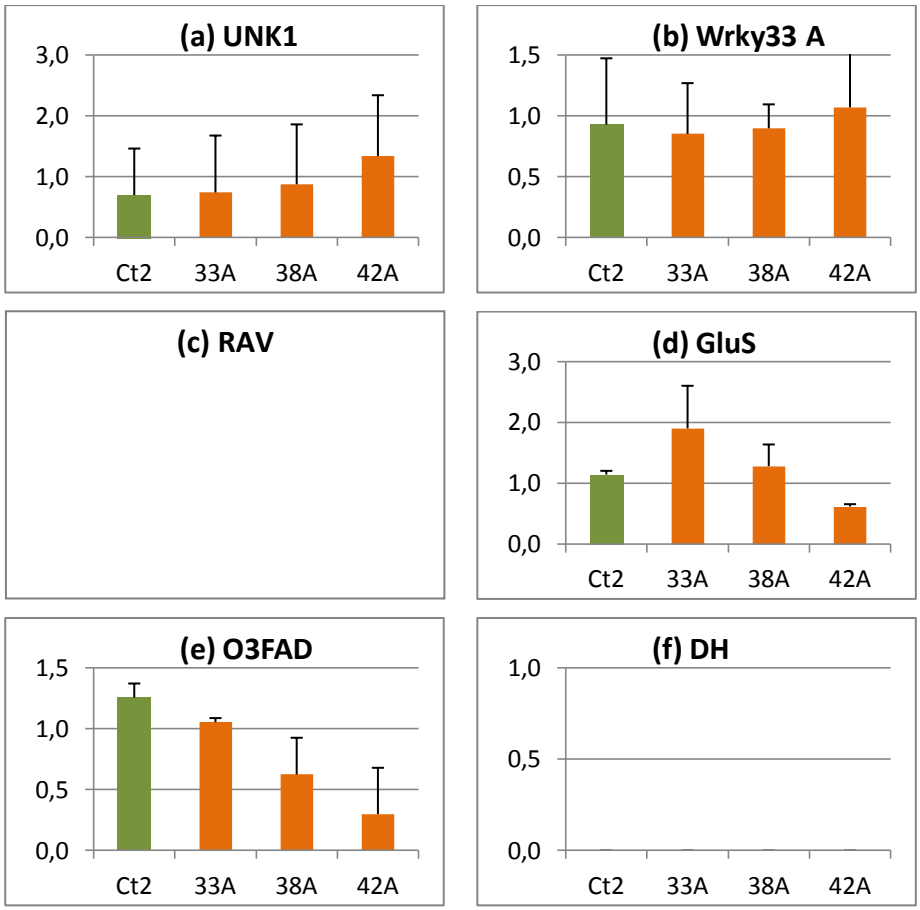


Figure 17 (a-p): Expression patterns of the putative genes of interest for the HSA samples (see Figures 13 and 15 and Table 7). Relative quantification of the RT-PCR using ImageJ software and normalizing all the genes to *QsActin*. Also represented the SD for each timepoint.



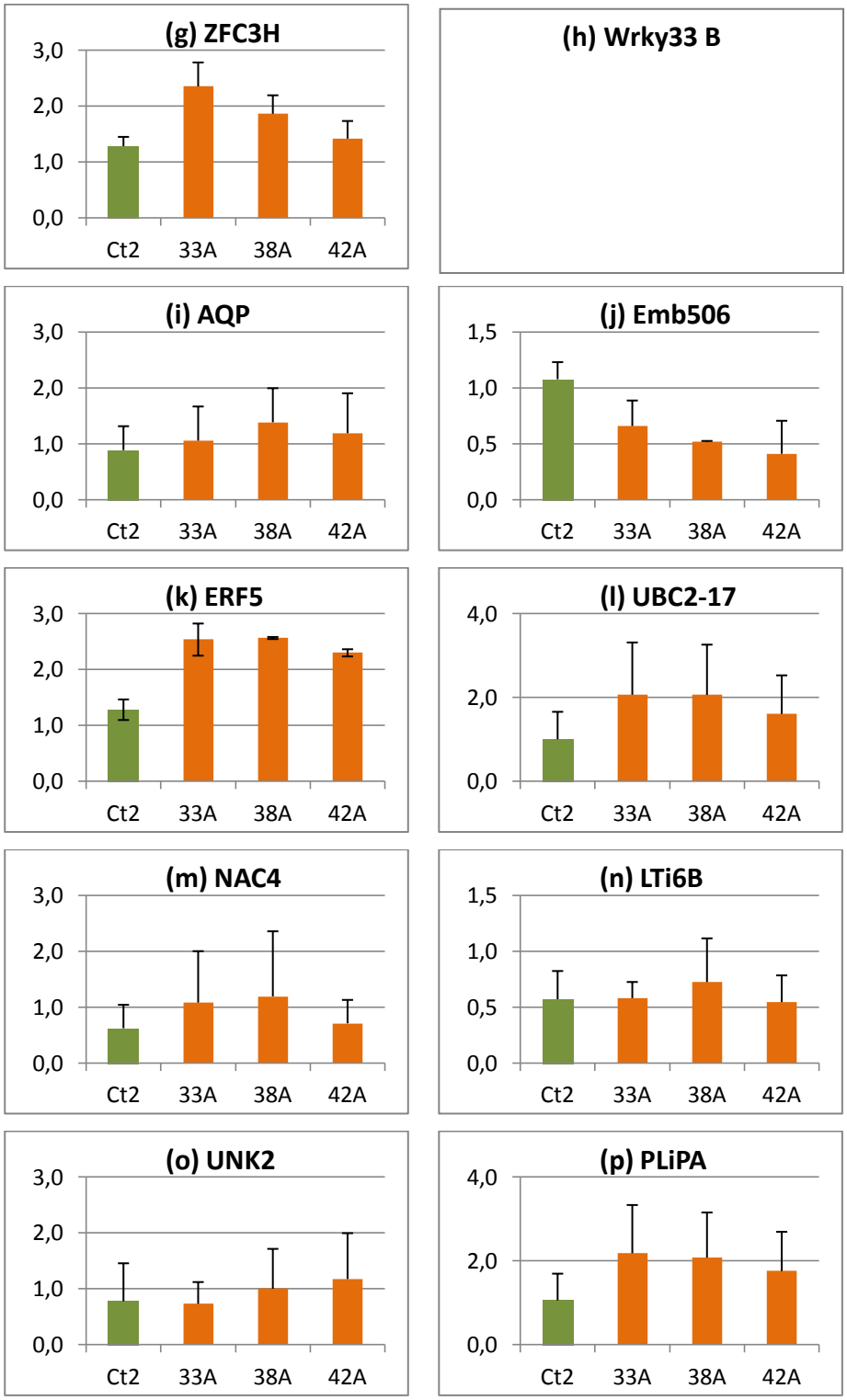


Figure 18 (a-p): Expression patterns of the putative genes of interest for the HAA samples (see Figures 13 and 15 and Table 7). Relative quantification of the RT-PCR using ImageJ software and normalizing all the genes to *QsProtSb*. Also represented the SD for each timepoint.

Table 8 summarizes the comparison between predicted and obtained expression patterns of the candidate genes in the three treatments.

Table 8: Comparison between the predicted and obtained expression pattern for the candidate genes for the cold stress (CSA), heat shock (HSA) and heat acclimation (HAA) samples.

Unigene	Predicted expression pattern	Expression pattern (RT-PCR)
QsAct	-	HKG (heat shock, cold)
QsProtSb	-	HKG (heat acclimation)
QsAQP	Upregulated by heat acclimation	Cold: - Heat: seems Upregulated by HS and HA
QsEMB506	Upregulated by heat	Cold: seems Upregulated by cold Heat: seems Downregulated by HA
QsDH	Upregulated by cold acclimation	Cold: seems Upregulated by cold (specially acclimation) Heat: only has expression at Ct5h
QsZFC3H	Upregulated by cold shock	Cold: seems Upregulated by cold Heat: seems Downregulated at HA (after peak at 33A)
QsRAV	Upregulated by cold shock	Cold: seems Upregulated by cold Heat: -
QsERF5	Upregulated by cold shock	Cold: seems Upregulated by CS Heat: seems Upregulated by HS and HA
QsUBC2-17	Upregulated by cold conditions	Cold: - Heat: seems Upregulated by HA
QsGluS	Downregulated by cold acclimation Downregulated by heat acclimation	Cold: seems Downregulated by cold Heat: seems Downregulated by HS and HA (after peak at 33A)
QsO3FAD	Downregulated by heat acclimation	Cold: - Heat: seems Downregulated by HS and HA
QsUNK1	Upregulated during heat acclimation	Cold: seems Upregulated by cold Heat: seems Downregulated by HS and Upregulated by HA
QsNAC4	Upregulated during heat acclimation	Cold: - Heat: seems to tend Up by HA
QsUNK2	Upregulated by cold acclimation	Cold: seems Upregulated by CA Heat: seems Downregulated by HS and tends Up by HA
QsWRKY33A	Upregulated by cold acclimation	Cold: seems Upregulated by CA Heat: seems Upregulated at Ct5h and tends Up by HA
QsWRKY33B	Upregulated by cold acclimation	Cold: seems Upregulated by cold Heat: seems to tend Down by HS
QsLTI6B	Upregulated by cold acclimation	Cold: - Heat: -
QsPLIPA	Upregulated by cold acclimation	Cold: seems Upregulated by CA Heat: seems Upregulated at HS and HA

From all the validated candidate genes, *QsEmb506*, *QsUBC2-17* and *QsLTi6B* obtained expression patterns contrary to the predicted. For some of the genes, no Up or Downregulation pattern can be observed (*QsLti6B*, *QsNac4*, *QsO3FAD*, *QsUBC2-17*, *QsRAV* and *QsAQP*) for at least one of the treatments.

The intensities of the bands, represented for each gene, are an average of the intensities obtained for two technical replicates. For that reason there is a great SD that can "mask" the expression pattern. All the patterns will be confirmed with biological replicates. Also, primers will be designed for the genes not yet tested. These genes will also be validated.

It is already being carried out a bibliographic research on the candidate genes. The expression data from the several stresses together with the bibliographic research, will lead to a selection of 10 of the 27 genes to proceed analysis by qRT-PCR.

Chapter 5. General conclusions and future perspectives

The main goal of this work was to assess the effect of thermal stress (specially heat stress) in cork oak plants. Previous studies showed that the trees can cope with high temperatures when allowed to acclimated (Correia *et al.*, 2011; Correia *et al.*, 2012), so a heat stress assay was performed with potted 5years-old cork oak plants in acclimation (gradual increase of temperature from 25-42ºC) and non-acclimation (heat shock of 42ºC for 5h) situations. Physiological data collected leads to the conclusion that, at the tested conditions, heat has no major effect on photosynthesis. That may be due to the expose time to the stress or the stress temperature not being enough to constitute a stronger challenge, as previous studies showed the plants can withstand higher temperatures for longer time (Correia *et al.*, 2012). A new stress assay will be performed to confirm physiological data observed and to collect new biological material (leaves). It would be important to collect additional data to confirm the plants' response to stress, for example calculate and control RWC, determine RuBisCO activity, electrolyte leakage and quantify chlorophyll a/b content (Correia *et al.*, 2011), as well as collect data from a larger tree population.

From previous stress assays, a group of non-normalized libraries of biotic and abiotic stresses transcripts were prepared. Within this work, total RNA was extracted and purified to construct the heat and cold stresses libraries. The *in silico* analysis of these libraries led to a list of 27 genes candidate as keyplayers in response to stress.

To the expression analysis, the RNA extraction protocol was optimized for the heat and cold stresses. From a list of several tested protocols, CTAB (Reid *et al.*, 2006) and Hot Borate (Wan and Wilkins, 1994) showed the best results for heat control and shock and heat acclimation and cold stress, respectively. Primers were designed and validated for 16 of the 27 candidate genes. The expression patterns of these genes were determined and will be confirmed with biological replicates; 13 of the 16 analyzed genes showed an expression pattern under cold and heat stress response as predicted by *in silico* expression analysis. Primers will also be designed and validated for the remaining genes. The comparison of all the genes' expressions for the several stresses will lead to a selection of 10 most promising genes for further analyzes by qRT-PCR.

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6. http://www.euforgen.org/ (EUFORGEN, European forest genetic resources programme web page)
7. http://www.fagaceae.org/ (Fagaceae genomics web page)

Additional Information

Appendix 1: Sigma's Spectrum plant total RNA kit™

1. Grind 100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Transfer the tissue powder to a frozen 2ml collection tube and add 500µl lysis buffer. Vortex vigorously for 30sec
2. Incubate the samples for 5min at 56ºC (optional)
3. Centrifuge for 4min @ max speed/rt.
4. Transfer the supernatant to the filtration column (blue column). Centrifuge for 2min @ 10000rpm/rt. Save the flow-through lysate
5. Pipette 750µl of binding solution into the lysate. Mix by pipetting 5 times.
6. Pipette 700µl of the mixture to the binding column (red column). Centrifuge for 2min @ max speed/rt.
7. Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper. Re-insert the red column in the collection tube and repeat step 8 with the remaining mix
8. Pipette 500µl of Wash Solution1 into column. Centrifuge for 2min @ max speed/rt. Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper. Re-insert the column in the collection tube.
9. Pipette 500µl of Wash Solution2 into the column. Centrifuge for 1min @max speed/rt Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper. Re-insert the column in the collection tube. Repeat step 9.
10. Centrifuge the column for 1min @ max speed/rt. Carefully discard the collection tube (avoid splashing)
11. Insert the column in new 2ml tube. Let rest with open lid for 1min (optional)
12. Pipette 50µl of elution solution on the center of the column's binding matrix. Close cap and let rest for 1min (optional)
13. Centrifuge for 1min @ max speed/rt to elute. The RNA is in the flow-through. Repeat steps 11-13.

Lysis buffer (Prepare fresh)

1. 500µl lysis buffer
2. 5µl β-mercaptoethanol (1%)

Appendix 2: Qiagen's RNeasy Plant Mini kit™

1. Grind 100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Transfer the tissue powder to a frozen 2ml collection tube and add 450µl lysis buffer. Vortex vigorously for 30sec
2. Optional step of 3min heat incubation @ 56ºC.
3. Transfer the lysate to the Qiashredder column (purple column in collection tube)
4. Centrifuge 2min @ max speed/room temperature (rt)
5. Transfer the supernatant to 1,5ml collection tube (without disturbing the pellet in the tube). May need to cut the tips' end
6. Add 0,5vol 100% ethanol and mix by pipetting. Do not centrifuge
7. Transfer all the sample, ±650µl, (with precipitates) to the MiniSpin column (pink column in collection tube)
8. Close lid gently and centrifuge 15sec @ 10000rpm/rt. Discard the flow-through
9. Add 700µl of RW1 buffer to the column. Centrifuge 15sec @ 10000rpm/rt to wash the column. Discard the flow-through
10. Add 500µl of RPE buffer to the column. Centrifuge 15sec @ 10000rpm/rt to wash the column. Discard the flow-through
11. Add 500µl of RPE buffer to the column. Centrifuge 2min @ 10000rpm/rt to wash the column.
12. Carefully transfer the column to a new 2ml collection tube. Centrifuge 1min @ max speed/rt to dry the column
13. Place the column in a new 1,5ml collection tube
14. Add 45µl (30-50µl) RNase-free water directly into the membrane of the column. Let rest for 1min with closed lid (optional). Centrifuge 1min @ 10000rpm/rt to elute RNA. The RNA is in the flow-through. Repeat step14 with RNase-free water or 30µl of the previous eluate.

Lysis buffer (Prepare fresh)

1. 450µl buffer RLC/ RLT
2. 4,5µl β-mercaptoethanol

Used lysis buffers RLC and RLT. For cork oak shows better results for buffer RLC.

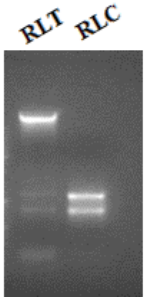

Figure 19: 1% agarose gel with ethidium bromide staining. RNA sample (1µg) extracted with RLT and RLC buffers. "RLC sample" clearly shows better results.

Appendix 3: Sigma's Spectrum plant total RNA™ kit (Brunner *et al.*, 2004)

1. Grind 100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N2. Transfer the tissue powder to a frozen 2ml collection tube and add 555µl lysis buffer. Vortex vigorously for 30sec
2. Incubate the samples for 5min at 56ºC (optional)
3. Add 0,4vol (222µl) Potassium Acetate (5M, pH 6,5) to the homogenate and mix by inversion. Incubate on ice for 15min
4. Centrifuge for 15min @ 12000rpm/4ºC
5. Transfer the supernatant to the filtration column (blue column). Centrifuge for 2min @ 10000rpm/rt. Save the flow-through lysate
6. Follow Sigma's Spectrum plant total RNA protocol (Appendix 1), starting in STEP 5.

Lysis buffer (Prepare fresh)

- 500µl lysis buffer
- 50µl 10% PVP40
- 5µl β-mercaptoethanol

Appendix 4: Qiagen's RNeasy Plant Mini kit™ (used for *Prunnus dulcis* (Brunner *et al.*, 2004))

1. Grind 100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid $_{N2.}$ Transfer the tissue powder to a frozen 2ml collection tube and add 500µl lysis buffer. Vortex vigorously for 30sec
2. Add 0,4vol (200µl) Potassium Acetate (5M, pH 6,5) to the homogenate and mix by inversion. Incubate on ice for 15min
3. Centrifuge for 15min @ 12000rpm/4ºC
4. Transfer the supernatant to the Qiashredder column (purple column in collection tube). Centrifuge 2min @ 10000rpm/room temperature (rt)
5. Follow Qiagen's RNeasy Plant Mini protocol (Appendix 2), starting in STEP 5.

Lysis buffer (Prepare fresh)

- 450µl buffer RLC/ RLT
- 45µl 10% PVP_{40}
- 4,5µl β-mercaptoethanol

Appendix 5: Zymo's Direct-zol RNA Miniprep™ kit

1. Grind 50mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Transfer the tissue powder to a frozen 2ml collection tube
2. Add 500µl of TriReagent and vortex vigorously to homogenize (I vortexed for 5min, which is too much)
3. Centrifuge for 3min @ 12000xg/rt and collect the supernatant to new 2ml tube
4. Add 1vol (±450µl) of 100% ethanol and my by vortex
5. Load the whole volume into Zymo-Spin IIC column in a new collection tube
6. Centrifuge 1min @ 12000xg/rt and discard the flow-through and the collection tube
7. Insert the column in a new collection tube. Add 400µl of Direct-zol RNA PreWash
8. Centrifuge for 1min @12000xg/rt and discard the flow-through. Repeat steps 7 & 8
9. Add 700µl of RNA Wash buffer. Centrifuge 1min @ 12000xg/rt and discard the flow-through and collection tube
10. Insert the column in a new collection tube. Centrifuge for 1min @ 12000xg/rt
11. Transfer the column to a 1,5ml tube. Add 30µl of RNase-free water
12. Centrifuge for 1min @ max speed/rt
13. Reload the flow-through in the column. Centrifuge for 1min @ max speed/rt
14. Perform the Turbo DNase treatment protocol and quantify (or in-column DNAse)

Appendix 6: Qiagen's RNeasy Plant Mini™ kit (used for *Jatropha curcas* (Gehrig *et al.*, 2000))

1. Grind 100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Transfer the tissue powder to a frozen 2ml collection tube and add 500µl lysis buffer. Vortex vigorously for 30sec
2. Incubate the samples for 5min at 56ºC (optional)
3. Add 0,4vol (200µl) Potassium Acetate (5M, pH 6,5) to the homogenate and mix by inversion. Incubate on ice for 15min
4. Centrifuge for 15min @ 12000rpm/4ºC
5. Transfer the supernatant to the Qiashredder column (purple column in collection tube). Centrifuge 2min @ 10000rpm/room temperature (rt)
6. Follow Qiagen's RNeasy Plant Mini protocol (Appendix 2), starting in STEP 5.

Lysis buffer (Prepare fresh)

- 450µl lysis buffer RLC
- 45µl 2% PEG HMW 20000
- 4,5µl β-mercaptoethanol

Appendix 7: Norgen's Plant/Fungi total RNA purification kit™

1. Grind 50mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Transfer the tissue powder to a frozen 2ml collection tube and add 600µl lysis buffer. Vortex vigorously for 30sec
2. Spin the lysate for 2min @600rpm
3. Centrifuge for 4min @ max speed/rt.
4. Transfer the supernatant to the filtration column (blue column). Centrifuge for 2min @ 10000rpm/rt. Save the flow-through lysate
5. Pipette 750µl of binding solution into the lysate. Mix by pipetting 5 times.
6. Pipette 700µl of the mixture to the binding column (red column). Centrifuge for 2min @ max speed/rt. Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper.
7. Re-insert the red column in the collection tube and repeat step 6 with the remaining mix
8. Pipette 500µl of Wash Solution1 into column. Centrifuge for 2min @ max speed/rt. Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper. Re-insert the column in the collection tube.
9. Pipette 500µl of Wash Solution2 into the column. Centrifuge for 1min @max speed/rt. Decant the flow-through liquid and tap the tube upside-down on a clean absorbent paper. Re-insert the column in the collection tube. Repeat step 9.
10. Centrifuge the column for 1min @ max speed/rt. Carefully discard the collection tube (avoid splashing)
11. Insert the column in new 2ml tube. Let rest with open lid for 1min (optional)
12. Pipette 50µl of elution solution on the center of the column's binding matrix. Close cap and let rest for 1min (optional)
13. Centrifuge for 1min @ max speed/rt to elute. The RNA is in the flow-through. Repeat steps 11-13.

Lysis buffer (Prepare fresh)

- 600µl lysis buffer
- 6µl β-mercaptoethanol (1%)

Appendix 8: CTAB RNA extraction Protocol for eppendorf (Reid *et al.*, 2006)

1. Grind 75-100mg of plant tissue o fine powder with pre-frozen mortar and pestle in liquid N_2. Pre-warm the extraction buffer at 65ºC.
2. Add the pre-warm buffer (1ml/ 100-500mg tissue sample). Vortex vigorously for around 30sec
3. Incubate the tubes in a 65ºC water bath for 10min. Shake every couple of minutes (or add to a water bath incubator at 150rpm)
4. Add 1ml of chloroform:isoamyl alcohol (24:1) and shake gently
5. Centrifuge at 13200rpm/10min @ 4ºC
6. Transfer the aqueous layer (upper) to a new tube. Repeat steps 4-6
7. Add 0,1volume of 3M NaOAc (pH 5,2) and 0,6volume of cold isopropanol to the supernatant. Mix gently and store at -80ºC/25min (1h)
8. Centrifuge at 13200rpm/30min @ 4ºC. Discard the supernatant
9. Dissolve the pellet in 250-375µl TE (pH 7,5)
10. Add 94-140µl of 8M LiCl to precipitate RNA. Incubate o/n @ 4ºC
11. Centrifuge at 13200rpm/30min @ 4ºC. Discard the supernatant
12. Add 1ml ice cold 70% EtOH. Centrifuge 0,5min and discard the supernatant
13. Let air dry in a flow-chamber (around 15-30min)
14. Dissolve in 50-150µl DEPC-treated water
15. Proceed with RNA cleanup protocol from RNeasy Mini kit™ (Qiagen)

CTAB isolation buffer:

- 2% hexadecyltrimethylammonium bromide [CTAB: Sigma H-5882],
- 1,4 M NaCl,
- 2% 2-mercaptoethanol,
- 20 mM EDTA,
- 100 mM Tris-HCl, pH 8.0

Appendix 9: Hot Borate Protocol (Wan & Wilkins, 1994)

1. Pre-warm a water bath at 80°C and turn on an incubator at 42ºC.
2. Pipette 10 ml of XT buffer for gram of plant tissue (fresh weight) to a Falcon 50ml tube. Add DTT, NP-40 & PVP-40 to XT buffer. Incubate at 80-90°C for 5-10 min.
3. Thaw proteinase K (stock solution 20 mg/ml, kept at –20°C) and place on ice.
4. Add the pre-warm buffer to the plant tissue powder (grinded in liquid N_2 with morta rand pestle). Vigorously homogeneize, vortexing for around 30sec.
5. Add (fast) 600 µl proteinase K for each 10 ml XT buffer. Incubate at 42ºC/150rpm for 1,5hours.
6. Add 800 µl KCl 2M for each 10 ml XT buffer (final dilution of 160 mM). Vortex and incubate on ice or at 4ºC for at least 1hour
7. Centrifuge at 10000 rpm (12000 g) for 25 min/ 4ºC.
8. Pipette the supernatant to a new tube, registering the volume.
9. Add ¼ vol of LiCl 8 M (to a final concentration of 2 M). Vortex and incubate at 4ºC o/n (LiCl precipitates selectively RNA instead of DNA).
10. Centrifuge for 20min/ 4ºC at 10000 rpm (12000 g). Discard the supernatant.
11. Observe the pellet.
    a. If it's clear, proceed to step 12
    b. If it's not clear, add 3-5ml cold LiCl 2M (4ºC). Centrifuge for 10min/4ºC at 10000 rpm (12000 g). Repeat if necessary.
12. Proceed with RNA cleanup protocol from RNeasy Mini kit™ (Qiagen)

Hot – Borate extraction Buffer (XT):

- 0,2 M Borax (MM = 381,4)
- 30 mM EGTA (MM = 380,4) (or EDTA)
- 1% (p/v) SDS
- 1% (p/v) sodium deoxycholate
- 10 mM DTT (MM = 154,3)*
- 1% (v/v) nonidet P-40 (NP-40)*
- 2% (p/v) PVP_{40} (MM = 40,0)*

* Add fresh

Appendix 10: Ambion's Turbo DNA-free kit™

(Optional: transfer samples to 0,5 or 1,5ml tubes (easier to collect the supernatant))

1. Adjust the sample volume to 50µl (10-100µl) with RNase-free water
2. Add 0,1vol of 10x turbo DNase buffer
3. Add 1µl DNase. Mix gently by flipping the tube
4. Incubate @ 37ºC/ 30min
5. Add 0,1vol of inactivation reagent. Before adding ressuspend the inactivation reagent by vortex
6. Mix well by vortex
7. Incubate for 5min @ room temperature (rt) vortexing occasionally
8. Centrifuge for 1,5min @ 10000xg/rt. Centrifuge only 2 samples @ time because step 9 must be fast
9. Carefully transfer the RNA supernatant to a new collection tube
10. Repeat steps 8 & 9 with the remaining samples

Appendix 11: Qiagen's RNeasy MinElute Cleanup kit™

1. Adjust sample volume to 200µl with RNase-free water (mix by gently vortex or pipetting)
2. Add 700µl buffer RLT and mix well by pipetting
3. Add 500µl of 100% ethanol and mix well by pipetting
4. (Fast!) Transfer 700µl of the mix to MiniSpin column (pink column in collection tube).
5. Centrifuge for 20sec @ 8220xg/rt. Discad the flow-through. Reinsert the column in the collection tube.
6. Repeat steps 4 and 5 with the remaining mix. Place column in new 2ml collection tube.
7. Add 500µl RPE. Centrifuge for 20sec @ 8220xg to wash the column's membrane. Discard the flow-through.
8. Add 500µl 80% ethanol to the column. Centrifuge for 2min @ max speed/rt to wash the membrane's column. Discard the flow-through and the collection tube
9. Place the column in a new 1,5ml tube. Add 14µl RNase-free water on the center of the column's matrix Centrifuge for 1min @ max speed/rt to elute 12µl RNA

Appendix 12: Total RNA Purification Protocol with PVP

1. Adjust sample volume to 400µl with RNase-free water (mix by gently vortex or pipetting)
2. Add 100µl of 10% PVP10 solution (ie, solution diluted to 2%) and mix by inversion.
3. Keep overnight (o/n) @ 4ºC
4. Add 1vol (500µl) chloroform:isoamyl alcohol (24:1) to the sample.
5. Vortex for 20sec and centrifuge for 20min @ 16000xg/4ºC
6. Transfer the upperphase to a new 2ml tube (attention do not transfer the middle and lower phases!)
7. Add 2vol (±900µl) of 100% ethanol and 0,1vol (±45µl) 5M NaCl solution
8. Precipitate for 2-3hours @ -80ºC
9. Centrifuge for 10min @ 16000xg/4ºC
10. Remove supernatant without touching RNA pellet (keep supernatant in ice for trouble-shooting)

From this step onwards use Qiagen's RNeasy Mini kit

11. Add 350µl RLT buffer and vortex 20 sec to ressuspend the pellet
12. Add 250µl 100% ethanol d mix by pipetting
13. Transfer the sample to MiniSpin column (pink column in collection tube). Centrifuge for 20sec @ 8000xg/rt and discard supernatant
14. Add 700µl of RW1 buffer to the column. Centrifuge for 20sec @ 8000xg/rt and discard the supernatant
15. Add 500µl of RPE buffer to the column. Centrifuge for 20sec @8000xg/rt and discard the supernatant
16. Add 500µl of RPE buffer to the column. Centrifuge for 2min @8000xg/rt and discard the supernatant. Let rest with the lid open for 1min
17. Place the column in new 1,5ml tube. Add 40µl of RNase-free water and centrifuge 1min @ 8000xg/rt to elute the RNA
18. Repeat step 16 twice with 30µl RNase-free water.

Appendix 13: Total RNA Purification Protocol with PVP (adapted)

1. Adjust sample volume to 400µl with RNase-free water (mix by gently vortex or pipetting)
2. Add 100µl of 10% PVP10 solution (ie, solution diluted to 2%) and mix by inversion.
3. Keep overnight (o/n) @ 4ºC
4. Add 1vol (500µl) chloroform:isoamyl alcohol (24:1) to the sample.
5. Vortex for 20sec and centrifuge for 20min @ 16000xg/4ºC
6. Transfer the upperphase to a new 2ml tube (attention do not transfer the middle and lower phases!)
7. Add 2vol (±900µl) of 100% ethanol and 0,1vol (±45µl) 5M NaCl solution
8. Precipitate for 2-3hours @ -80ºC
9. Centrifuge for 12min @ 16000xg/4ºC
10. Remove supernatant without touching RNA pellet (keep supernatant in ice for troubleshooting)
11. Add 500µl of 70% ethanol (cold). Centrifuge for 12min @ max speed/4º-C. Discard the supernatant carefully tapping the tube upside-down in a clean absorbent paper
12. Dry the pellet in the flow chamber (15-20min)
13. Ressuspend the RNA in 25-50µl RNase-free water

Appendix 14: Ethanol/NaOAc Precipitation Protocol

1. Add 0,1vol of 3M NaOAc (pH 5,2) solution
2. Add 2,5vol of 100% ethanol (cold)
3. Mix gently by inversion. Incubate o/n @ -20ºC
4. Centrifuge for 30min @ 11000rpm/4ºC
5. Remove the supernatant.
6. Wash the pellet with 500µl 75% ethanol (cold)
7. Centrifuge for 15min @ 11000rpm/4ºC
8. Discard the supernatant carefully tapping the tube upside-down in a clean absorbent paper
9. Dry the pellet in the flow chamber (15-20min)
10. Ressuspend the RNA in 25-50µl RNase-free water

Appendix 15: Potassium Acetate Precipitation Protocol

(Sample ressuspended in 100-200µl TE or RNase-free water)

1. Add 1vol of 2M KOAc (pH 4,8) solution. Incubate on ice for 15min
3. Centrifuge for 20min @ 12000xg/4ºC
4. Collect the supernatant
5. Add 2,5vol of 100% ethanol (cold) to precipitate the RNA
6. Mix gently by inversion. Incubate for 15 min or o/n @ -20ºC
7. Centrifuge for 20min @ 12000xg/4ºC
8. Discard the supernatant carefully
9. Wash the pellet with 600µl 75% ethanol (cold)
10. Centrifuge for 10min @ 12000xg/4ºC
11. Discard the supernatant carefully tapping the tube upside-down in a clean absorbent paper
12. Dry the pellet in the flow chamber (15-20min)
13. Ressuspend the RNA in 25-50µl TE or RNase-free water

Appendix 16: Qiagen's RNeasy Mini kit™

1. Adjust sample volume to 100µl with RNase-free water (mix by gently vortex or pi-petting) .Add 350µl buffer RLT and mix well by pipetting
2. Add 250µl of 100% ethanol and mix well by pipetting
3. (Fast!) Transfer the sample (700µl) to MiniSpin column (pink column in collection tube). Centrifuge for 20sec @ 8220xg(10000rpm)/rt. Discad the flow-through. Re-insert the column in the collection tube.
4. Add 500µl RPE. Centrifuge for 20sec @ 8220xg to wash the column's membrane. Discard the flow-through.
5. Add 500µl RPE. Centrifuge for 2min @ 8220xg to wash the column's membrane. Discard the flow-through.
6. Place the column in a new 2ml tube. Centrifuge at max speed for 1min
7. Place the column in a new 1,5ml tube. Add 30-50µl RNase-free water on the center of the column's matrix. Let the tube rest for 1min.
8. Centrifuge for 1min @ max speed/rt to elute the RNA
9. Repeat steps 7-9 with 30-50µl RNase-free water or the flow-through.