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Tese de Doutoramento em Ciências
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Trabalho realizado sob a orientação da
Prof. Doutora Teresa Lino-Neto

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Characterisation of *Arabidopsis thaliana* Heat-Responsive RNA-Binding Protein (*HRR*) Gene: Molecular Roles in Plant Thermotolerance and Development

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Characterisation of *Arabidopsis thaliana Heat-Responsive RNA-Binding Protein* (*HRR*) Gene: Molecular Roles in Plant Thermotolerance and Development

ABSTRACT

Plants face a multiplicity of biotic and abiotic stresses, of which the most typical are extreme temperatures. High temperatures cause considerable morphological, physiological and molecular alterations that adversely affect plant growth and productivity. The acquisition of thermotolerance is controlled by activation and regulation of specific stress-related genes that lead to adjustments on plant transcriptome and proteome. From a previous transcriptomic analysis of heat-stressed Arabidopsis seedlings, the *HRR* gene was singled out by presenting a specific high heat-stress response. The principal aim of this work is the functional characterisation of this uncharacterized gene. At the end, this work is expected to contribute for the general understanding of RNA-binding proteins involvement in heat stress responses. These proteins are expected to be associated to the transcriptome organisation responsible for adaptation to heat stress, as well during plant development.

The *in silico* analysis revealed that *HRR* codes for a putative RNA-binding protein, containing a RRM domain and a PABP-1234 functional domain. *HRR* was predicted to be highly expressed under heat stress conditions. However, *HRR* seems to present a basal expression throughout the plant life cycle, being prevised the highest levels during flower development, seed maturation and germination. The predicted co-interaction with other Arabidopsis RRM-containing proteins (UBP1 and RBP45 proteins) and phylogenetic relationship with metazoan orthologues suggests that HRR could play functions in the stability of HS-induced transcripts.

The phenotypic analysis of *hrr* loss-in-function and HRR over-expression mutant lines showed that HRR seems to be strongly involved in plant thermotolerance responses, at least during seed germination. In opposition to bioinformatic data, HRR appears to be also involved in responses to salt stress imposition. Furthermore, HRR was suggested to be a positive regulator in the metabolism and signalling of phytohormone ABA.

When seedlings were subjected to heat stress, the *HRR* expression analysis revealed that *HRR* transcripts are subjected to alternative splicing process, originating the canonical *HRR.1* and intron-retained *HRR.2* transcripts. A mRNA decay analysis suggested that *HRR.2* transcripts could

be considered good targets for RNA degradation, most likely through nonsense mRNA decay mechanisms. The alternative splicing mechanism was not always evident. In seeds, either subjected or not to heat stress, *HRR.1* was the only transcript to be originated. Therefore, depending on plant development stage, HRR proteins could display slightly different functional roles. HRR proteins appear to be crucial in the regulation of other heat stress-responsive transcripts (*HSFs* and *HSPs*). In agreement with bioinformatic analysis, *HRR* is expressed during the later stages of seed maturation and during transition from seed dormancy to germination phases. During these stages, HRR seems to modulate a set of seed-specific transcripts, namely *ABI5*, *Em6*, *HSFA9* and *HSP101.* ABA biosynthesis (*ABA1* and *NCED9*) and *SPY* (GA metabolism negative regulator) transcripts seem to be also regulated by HRR, during seed germination.

In vitro localisation assays suggested that HRR proteins appear to follow distinct subcellular pathways during HS imposition. Initially, HRR.1 was found in the nucleus, being then recruited for cytoplasmic granules and nuclear pores. HRR.2 was mainly found in cytoplasmic granules but was also present in nuclear speckles. The localisation of both proteins in cytoplasmic aggregates suggests that they could be present in stress granules (SGs) and/or processing bodies (PBs). Transcriptional- and translational-inhibition experiments demonstrated that HRR.1 could be strongly involved in SG biogenesis, while HRR.2 could interfere in both SG and PB activities.

The approaches used in this work to investigate the HRR function have disclosed the role of this protein in heat-stress responses and during seed development and germination. Further research on these proteins will strength the current knowledge about the RNA metabolism under heat stress conditions. However, the key features of plant RNA-binding proteins in abiotic stress responses and plant development have just begun to be uncovered and many questions remain still to be answered.

Caraterização do gene *HRR* (*Heat-Responsive RNA-Binding Protein*) em *Arabidopsis thaliana*: Funções Moleculares na Termotolerância e Desenvolvimento Vegetais

RESUMO

As plantas estão continuamente a ser sujeitas a uma multiplicidade de stresses bióticos e abióticos, sendo que as temperaturas extremas são a forma mais comum de stresse abiótico. As temperaturas elevadas provocam consideráveis alterações morfológicas, fisiológicas e moleculares nas plantas, as quais afetam negativamente o seu crescimento e desenvolvimento. A aquisição de termotolerância é efetuada pela ativação e regulação de genes específicos para a resposta ao stresse, conduzindo a ajustamentos no transcriptoma e proteoma da planta. Da prévia análise transcriptómica efetuada em plântulas de *Arabidopsis* sujeitas a stresse pelo calor, o gene *HRR* foi selecionado por apresentar uma específica e elevada resposta ao stresse pelo calor. O principal objetivo deste trabalho é a caracterização funcional deste gene de função desconhecida. No final deste trabalho espera-se que a caracterização funcional de HRR contribua para o maior conhecimento da funcionalidade das proteínas de ligação ao RNA nas respostas ao stresse pelo calor. Estas proteínas provavelmente estão associadas à re-organização do transcriptoma, a qual será responsável pela adaptação ao stresse pelo calor e em diferentes fases do desenvolvimento vegetal.

A análise *in silico* revelou que o gene *HRR* codifica para uma putativa proteína de ligação ao RNA, sendo particularmente expresso sob condições de stresse pelo calor. No entanto, *HRR* parece apresentar uma expressão basal ao longo de todo o ciclo de vida da planta, estando previstos os níveis mais elevados durante o desenvolvimento floral, maturação das sementes e germinação. A previsão da co-interação de HRR com outras proteínas de Arabidopsis contendo o domínio RRM (proteínas UBP1 e RBP45) e a sua relação filogenética com ortólogos de metazoários sugere que HRR pode desempenhar funções na estabilidade de transcritos induzidos durante o stresse pelo calor.

A análise fenotípica de linhas mutantes de HRR com perda- (*hrr*) e ganho-de-função (sobreexpressão) demonstrou que HRR pode estar fortemente envolvida nas respostas de termotolerância, pelo menos durante a germinação das sementes. Em oposição aos dados bioinformáticos, HRR parece também estar envolvido nas respostas ao stresse salino. Foi

igualmente sugerida a função de HRR como regulador positivo no metabolismo e sinalização da fitohormona ABA.

Quando plântulas foram submetidas ao stresse pelo calor, a análise de expressão de HRR revelou que os transcritos de HRR são sujeitos a um processo de excisão alternativa, originando o já descrito transcrito *HRR.1* e o transcrito *HRR.2* que apresenta retenção de um intrão. A análise de decaimento de mRNA sugeriu que os transcritos de *HRR.2* podem ser considerados potenciais alvos de degradação, provavelmente através de mecanismos de decaimento de mRNA *nonsense*. O mecanismo de excisão alternativa nem sempre é verificado. Nas sementes, quer sejam sujeitas ou não ao stresse pelo calor, o único transcrito produzido é *HRR.1*. Deste modo, dependendo da fase de desenvolvimento, as proteínas HRR poderão apresentar ligeiras diferenças funcionais.

As proteínas HRR parecem ser importantes para a regulação de transcritos induzidos durante a resposta ao stresse pelo calor (*HSFs* e *HSPs*). De acordo com a análise bioinformática, *HRR* é expresso durante as últimas fases da maturação das sementes e durante a transição do estado de dormência para a germinação. Durante estas fases, HRR parece modular um grupo específico de genes, nomeadamente *ABI5*, *Em6*, *HSFA9* e *HSP101*. Os transcritos para enzimas envolvidas na biossíntese de ABA (*ABA1* e *NCED9*) e de *SPY* (regulador negativo no metabolismo do GA) parecem também ser regulados por HRR durante a germinação.

Ensaios *in vitro* de localização subcelular sugerem que as proteínas HRR seguem vias subcelulares diferentes, durante a imposição de stresse pelo calor. Inicialmente, HRR.1 foi encontrada no núcleo, sendo depois recrutada para grânulos citoplasmáticos e poros nucleares. HRR.2 foi maioritariamente encontrada nos grânulos citoplasmáticos, estando também presente em agregados subnucleares. A localização das duas proteínas nos agregados citoplasmáticos sugere que ambas estão presentes em grânulos de stresse (SGs) e/ou corpos de processamento (PBs). Ensaios de inibição da transcrição e tradução sugerem que HRR.1 está fortemente envolvida na biogénese de grânulos de stresse, enquanto HRR.2 pode interferir na atividade de ambos os tipos de agregados citoplasmáticos.

As abordagens utilizadas neste trabalho para estudar a função de HRR revelaram a função desta proteína nas respostas ao stresse pelo calor e durante o desenvolvimento das sementes e germinação. Trabalhos futuros sobre estas proteínas permitirão reforçar o conhecimento atual sobre o metabolismo do RNA em condições de stresse térmico pelo calor. Contudo, as características-chave das proteínas de ligação ao RNA nas respostas ao stresse abiótico e desenvolvimento vegetal só começaram agora a ser desvendadas e muitas questões permanecem ainda por responder.

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ABBREVIATIONS AND ACRONYMS

A	Absorvance (nm)
ABA	Abscisic acid
ABI	Abscisic acid insensitive
ACC	1-aminocyclopropane-1-carboxilic acid
ACT2	Actin2
ActD	Actinomycin D
AGRIS	Arabidopsis Gene Regulatory Information Server
ANOVA	Analysis of variance
AS	Alternative splicing
AT	*Arabidopsis thaliana*
AtcisDB	Arabidopsis *cis* regulatory elements database
Athena	*Arabidopsis thaliana* expression network analysis
BAR	Bio-Array Resource
BP	BP recombination (Gateway)
bp	Base pair
BSA	Bovine Serum albumin
BY2	Bright Yellow 2 cells
C	Carboxyl terminus
CaM	Calmodulin
CBC	Cap binding complex
CBK	CaM-binding protein kinase
CBL	Calcineurin B-like protein
CBP	Cap binding protein
cDNA	Complementary DNA
CDS	Coding sequence
CHX	Cycloheximide
CIPK	CBL-interacting protein kinase
CML	CaM-like protein
CTAB	Hexadecyltrimethylammoniumbromide
DAPI	4'-6-Diamidino-2-phenylindole
DCL	Dicer-like protein
DCP	Decapping protein
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DREB	Dehydration-responsive element binding
DS	Donor site
dsRBD	Double stranded RNA-binding domain
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic translation-initiation factors
EIN	Ethylene insensitive
EJC	Exon junction complex
eRF	Eukaryotic release factors
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
EST	Expressed Sequence Tags
ETR	Ethylene receptor
FUS3	FUSCA3
Fwd	Forward
g	Gram
g	Gravity force (relative centrifugal force)
GA	Gibberelic acid or gibberelin
GAI	GA insensitive
GAox	Gibberellin oxidase
GFP6	Green fluorescence protein (6)
GID1	GA-insensitive Dwarf1
GR-RBP	Glycine-rich RNA-binding protein
GT	Gene trap
GUS	*β* -glucuronidase
h	Hour
HLY1	*Hyponastic leave 1*
hnRNP	Heterogenous ribonucleoprotein
HRR	Heat responsive RNA-binding protein
HS	Heat stress/ high temperatures
HSA	Heat stress-associated
HSE	Heat shock element
HSF	Heat shock transcription factor
HSG	Heat stress granule
HSP	Heat shock protein
IAA	Isoamyl alchool
IP3	D-myo-inositol-1,4,5-triphosphate
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
JIC	John Innes Centre
Kb	Kilobases
KGG	Arg-Gly-Gly box
KH	K homology
LB	Left border
LEA	Late embryogenesis abundant protein
LEC	LEAFY COTYLEDON
Ler	*Landsberg erecta*
LP	Left primer
LR	LR recombination (Gateway®)
M	molar
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino) ethanesulfonic acid
min	Minute
miRNA	Micro RNA
mol	mole
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MS	Murashige-Skoog medium
N	Amino terminus
n	Nano
NASC	Nottingham Arabidopsis Stock Centre
NCBI	National Center for Biotechnology Information

NCED	9-cis-epoxycarotenoid dioxygenases
NMD	Nonsense-mediated mRNA decay
ºC	Degree Celsius
OD	Optical density
Os	*Oryza sativa*
p*35S*	Constitutive *35S CaMV* promoter
PA	Phosphatidic acid
PABP	Poly(A) binding protein
PAZ	Piwi/Argonaute/Zwille
PB	P-body
PCR	Polymerase chain reaction
pDEST	Destination vector (Gateway)
pENTR	Entry clone
Pfu	*Pirococcus furiosus*
p*HRR*	*HRR* promoter
PhyA/B	Phytochrome A/B
PIP2	Phosphatidyl 4,5-biphosphate
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PLD	Phospholipase D
pre-mRNA	Pre-messenger RNA
PTC	Premature termination codon
PUF	Pumilio/FBF
PUR	Puromycin
QTL	Quantitative trait loci
RB	Right border
RBM	RNA binding motif
RBP	RNA binding protein
RGA	Repressor of ga1-3
RGL1	RGA like-1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
Rnase	Ribonuclease
RNP	Ribonucleoprotein domain
ROS	Reactive oxygen species
RP	Right primer
rpm	Rotations per minute
RRM	RNA recognition motif
RT-PCR	Reverse transcriptase-polymerase chain reaction
Rv	Reverse
SAP	Shrimp Alkaline Phosphatase
SDS	sodium dodecyl sulphate
sec	Second
SEM	Standard error of mean
SG	Stress granule
siRNA	Small interference RNA
snRNP	Small nuclear ribonucleoprotein
SR	Serine/arginine rich
SSP	Seed storage protein
T2	Transformant generation
TAE	Tris-acetate-EDTA buffer
TAIR	The Arabidopsis Information Resource
Taq	*Thermophilus aquaticus*
TF	Transcription factor
TILLING	Targeting Induced Local Lesions in Genome
U	unit
UBA	UBP1-associated protein
UBP	U-rich binding protein
UCE	Ultra-conserved elements
UTR	Untranslated region
UV	Ultra-violet radiation
V	Volts
v/vol	Volume
ZnF	Zinc finger

A	Adenine
C	Cytosine
G	Guanine
T	Thyamine
U	Uracil
R	A or G (Purine)
Y	C or T(Pyrimidine)
ATP	Adenosine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycitidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythimidine triphosphate
dNTP	Deoxynucleotide triphosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphospahate

A	**Ala** Alanine
C	**Cys** Cysteine
D	**Asp** Aspartic acid
E	**Glu** Glutamic acid
F	**Phe** Phenylalanine
G	**Gly** Glycine
H	**His** Histidine
I	**Ile** Isoleucine
K	**Lys** Lysine
L	**Leu** Leucine
M	**Met** Metionine
N	**Asn** Asparagine
P	**Pro** Proline
Q	**Gln** Glutamine
R	**Arg** Arginine
S	**Ser** Serine
T	**Thr** Threonine
V	**Val** Valine
W	**Trp** Tryptophane
Y	**Tyr** Tyrosine
X	——— Unspecific amino acid

PUBLICATIONS

Azevedo H, Silva-Correia J, Oliveira J, Laranjeira S , Barbeta C, Amorim-Silva V, Botella MA, Lino-Neto T, Tavares RM. (**2011)**. A strategy for the identification of new abiotic stress determinants in Arabidopsis using web-based data mining and reverse genetics. ***OMICS: A Journal of Integrative Biology*** 15(12):935-947. (*Special Issue: Abiotic Stress in Plants*) (DOI 10.1089/omi.2011.0083)

Oliveira J, Tavares RM, Lino-Neto T. HRR, a novel RNA-binding protein involved in Arabidopsis thermotolerance responses (manuscript in preparation)

Oliveira J, Azevedo H, Tavares RM, Lino-Neto T. "**Functional characterisation of a putative post-transcriptional factor involved in *Arabidopsis thaliana* thermotolerance**", XXXV Portuguese Genetic Journeys, 31 May- 2 June 2010, Braga, Portugal (Poster)

Oliveira J, Azevedo H, Tavares RM, Lino-Neto T. "**Identification of a putative post-transcriptional regulator implicated in *Arabidopsis thaliana* thermotolerance**", Plant Genomics European Meetings, 7-10 October 2009, Lisbon, Portugal (Poster)

Oliveira J, Correia J, Azevedo H, Tavares RM, Lino-Neto T. "**Analysis of thermotolerance molecular regulation in A*rabidopsis thaliana* by reverse genetics approach", XVI National Congress of Biochemistry,** 22-25 October 2008, Ponta Delgada, Portugal (Poster)

1. GENERAL INTRODUCTION

1.1 Plant abiotic stress - their impact in modern agriculture

In response to an increasing world population and constant necessity of food supply, modern agriculture has been facing considerable challenges. The techniques used in modern agriculture have demonstrated limitations in substantially increasing crop productivity, mostly due to the adverse effects of stress imposed by environmental changes. For instance, 51-82% of potential yield of annual crops is estimated to be lost in developing countries (Nagarajan and Nagarajan 2010). To reduce the losses in crop productivity and avoid a progressive food shortage, a collective effort in plant science research has been carried out, in order to understand plant adaptations to environmental stresses.

Plants are susceptible to abiotic and biotic stresses. Abiotic stresses are characterised by a physical or chemical input, while biotic stresses are caused by interacting organisms (pathogens, predators and other competing organisms) (Robert-Seilaniantz *et al.* 2010). Drought and temperature are the major abiotic stresses that affect plants, along with salinity, light intensity and nutrient stress. These stresses can act simultaneously and increase the pressure over plants. For example, an increase in salt content of soil due to water loss is frequent during drought periods. These stresses, combined with intermittent non-optimal temperatures can substantially reduce crop production in many parts of the world (Mittler 2006).

To cope with abiotic and biotic stresses, plants have been developed a broad range of mechanisms and strategies to ensure their prevalence under stressful conditions. The impact in plant physiology is greatly determined by the intensity and duration of single or combined stresses. Plant susceptibility, genotype and structure also influence the survival of plants under stress conditions. Thus, the knowledge about mechanisms associated to plant resistance to stressful environments has been the central aim for abiotic stress research. This knowledge would be used to develop new crops with enhanced tolerance to abiotic stresses.

In recent years, the development of numerous methodologies and molecular tools have promoted the understanding of perception mechanisms and signalling responses to abiotic stress, mainly orchestrated by the expression of hundreds of genes. The identification and functional characterisation of genes involved in enhancing stress tolerance has been performed through transgenic lines (T-DNA, RNAi and TILLING mutants). Recent advances in microarray technology, functional genomics and development of high-throughput proteomics and metabolomics allowed the discovery of the molecular role of many stress-induced genes (Mittler and Blumwald 2010).

Basic research on plant responses to abiotic stress has been carried out on plant models and further transferred to crops of high economical interest. In addition to rice, *Arabidopsis thaliana* has been extensively used as a plant model for functional studies and has been considered very important to applied research (MASC 2011). Therefore, the basic Arabidopsis research functions as a pivotal tool to study plant stress biology. The knowledge obtained with these studies will allow to reduce the negative effects of environmental stress in crops, promoting plant productivity and ultimately reducing the worldwide food shortage.

1.2 The role of the model plant *Arabidopsis thaliana* in functional genomics

The study of plant stress has tended to focus on crop and wild species that develop a high adaptation ability to abiotic stress. The existence of crop variants displaying to specific trait(s) of stress tolerance has been of crucial importance, to the understanding of the genetic mechanisms underlying plant stress responses. The selection of a model system suitable for studying important processes common to all plants is another strategy to get the fundamental knowledge of such plant tolerance mechanisms. The *Arabidopsis thaliana* (Arabidopsis) (Figure 1.1) is a small plant belonging to Brassicaceae family, is native to Europe, Asia and north-western Africa, being also distributed throughout North America. Many ecotypes have been chosen from natural populations to be experimental by analysed (Table 1.1). Currently, the ecotypes Columbia and *Landsberg erecta* have been accepted as standards for genetic and molecular studies.

Table 1.1 Origin of the main *Arabidopsis thaliana* ecotypes, used in plant biology studies (http://nasc.nott.ac.uk/; http://www.arabidopsis.org/)

Ecotype	Origin
Col-0 (Columbia)	United States of America
Ler (*Landsberg erecta*)	Poland
WS (Wassilewskija)	Russia
Cvi-0	Cape Verde Islands

Arabidopsis has been considered as the main plant model for a number of reasons. This was the first plant species having the genome entirely sequenced in (Arabidopsis Genome Initiative 2000). Arabidopsis possesses a small genome (~120 Mbp; 25,500 genes) supported into five

chromossomes (Huala *et al.* 2001). Due to genomics traits and being a diploid organism, Arabidopsis has been suitable and easy for genetic manipulation: easily transformed by Agrobacterium and has a number of T-DNA lines, cDNA clones, TILLING and RNAi lines (Kuromori *et al.* 2009).This small angiospermic possesses a relatively small life cycle (~ six weeks) *and* generates a high number of seeds (~20,000 seeds per plant). Although being self-fertile and diploid, plants can be crossed by applying pollen to the stigma surface (Meinke *et al.* 1998). Considering these reasons and the very extensive information existent from different genetic resources, this plant species becomes a reference tool for stress research.

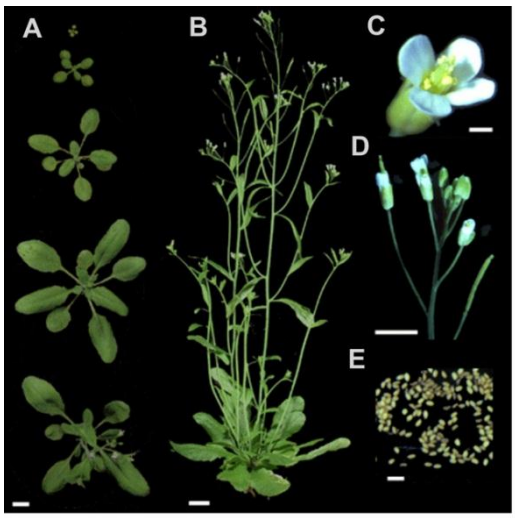


Figure 1.1 *Arabidopsis thaliana* - reproductive and vegetative development stages. This plant is a model broadly used in biochemical, physiological and molecular studies. (A) vegetative development before flowering; (B), adult plant; (C), flower, (D) floral stem and (E) seeds. White bars, 1cm, except in seeds (1 mm) Adapted from: http://wwwijpb.versailles.inra.fr/en/sgap/equipes/cyto/arabido.htm].

Even without agricultural value and not exhibiting unusual stress-tolerance, Arabidopsis importance lies on the discovery of gene and protein functions and in the previous knowledge on its plant physiology, morphology, metabolism and development (Meinke *et al.* 1998). At the time of completion of the genome sequence, only ~10% of the 25,500 genes initially predicted had an experimentally assigned function. Although being a tremendous challenge, for determining the function of remaining 90% of genes, the complementation between the structural and functional genomics approaches becomes essential (Alonso and Ecker 2006).

In the early stage of genome analysis, the structural genomics establish the genetic and physical mapping of an organism, as well as its EST libraries. The functional genomics analysis promotes the knowledge of gene function through the structural genomics data, as well as the information obtained from bioinformatic tools. In recent years, the functional genomics has been performed through forward genetics and, most intensively, through reverse genetics (Figure 1.2) (Alonso and Ecker 2006; Feng and Mundy 2006).

The primary tool available to functional genomics studies was forward genetics screens, in which the principal aim is to identify a mutation that produces a certain phenotype (Feng and Mundy 2006). Initially, a mutant population is generated by chemical (EMS, ENU), physical (UV, X-ray, fast neutron) or biological (transposon, T-DNA) mutagenesis. This approach enables genome "saturation" in which all potential genes can be mutated. This is followed by a screening in specific conditions to find a plant with the desired phenotype. Once identified the plant exhibiting the phenotype, a map-based cloning is performed to identify the genetic cause of the mutant phenotype (Alonso and Ecker 2006; Feng and Mundy 2006).

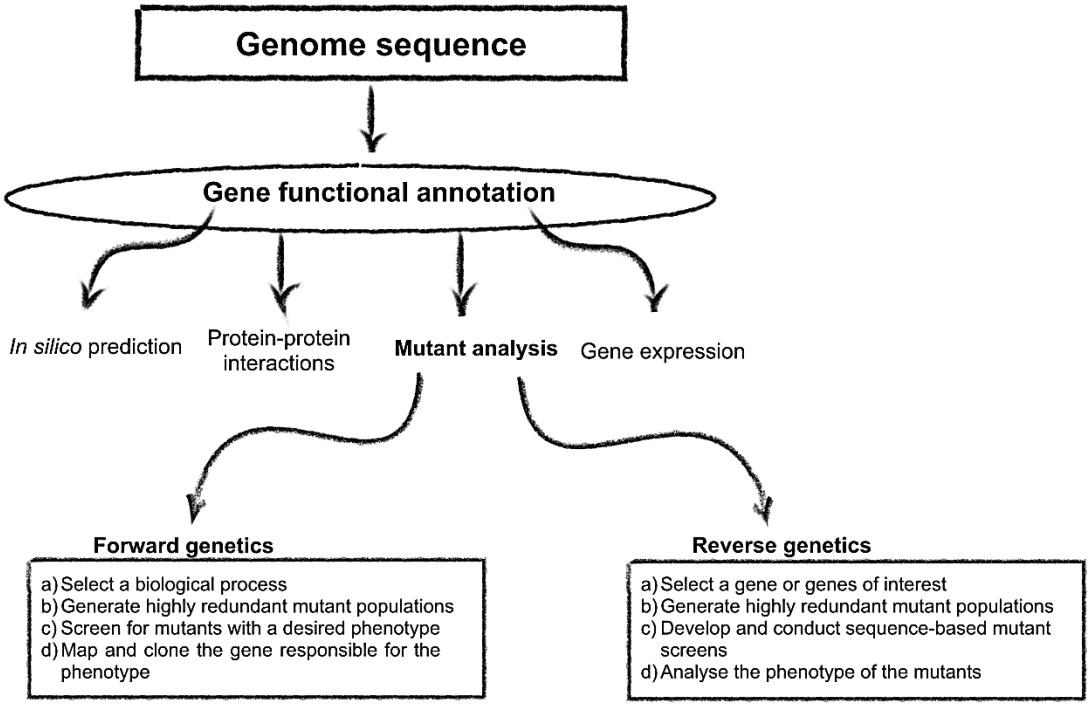


Figure 1.2 Schematic representation describing functional annotation for a gene, using either the forward or reverse genetics approaches. The most recent methodologies used in functional genomics are based on DNA chips, protein-protein interactions, analysis of expression profiles and available mutants. Two approaches can be used: forward genetics (identification and genomics mapping of a mutation which promotes a phenotype) and reverse genetics (determination of phenotype from a mutation into a gene of interest). Adapted from Alonso and Ecker (2006).

In opposition, reverse genetics attempts to find the phenotype that results from a specific mutated gene. Following this approach, the mutants can be obtained through RNAi, T-DNA and transposon insertional mutagenesis, Deleteagene or TILLING. The existence of a knockout line for the gene of interest is crucial to determine the effect of this gene in a specific biological process (Krysan *et al.* 1999; Feng and Mundy 2006). The majority of used knockout lines harbour a T-DNA-tagged insertion [corresponding to a portion of *Agrobacterium tumefaciens,* tumor-inducing (Ti) plasmid]. Owing to its disruptive nature, T-DNA insertion mutagenesis is commonly associated with

loss-in-function. However, it can be adapted to generate gain-of-function alleles by activation tagging. To achieve this, a strong transcriptional enhancer is introduced into the T-DNA, causing the ectopic expression of the nearby gene. Alternatively, the transposon-based insertion lines have demonstrated to be the most sophisticated approach in reverse genetic studies. Transposable elements are found in almost all organisms and are the major agents for generating diversity through mutation. Once considered potential mutagenesis agents, they have been exploited in reverse genetics approaches. Besides interrupting genes, additional refinements can show how the interrupted genes are expressed, or even produce gain-in-function phenotypes. This can be achieved by the use of engineered insertion elements, enhancer or gene traps. The main disadvantage of transposon tagging corresponds to one of the advantages of T-DNA insertion lines: the chemical and physical stability of genome integration through multiple generations (Krysan *et al.* 1999; Alonso and Ecker 2006; Feng and Mundy 2006).

Nowadays, much information has been provided from different methodologies, though many stress-responsive gene functions remain elusive. Both forward and reverse genetic approaches are important for elucidating gene functions but, progressively, reverse genetic has been the predominant methodology. Considering the organism and the biological trait to be analysed, as well as the access to correspondent insertion lines, the reverse genetics became the better strategy to integrate associated biological functions to heat stress (HS)-induced genes.

1.3 Temperature stress - the major threat for plants

Temperature is one the most important environmental factors that regulate plant growth and development. Each plant species display a range of optimal temperatures, which promotes the normal plant development (Saidi *et al.* 2011). The stress situation associated to high or low temperatures has a tremendous impact on all aspects of plant development and growth. In order to predict plant ability to adapt to environmental conditions that are permanently changing, the determination of optimal temperatures and identification of important components involved in responses to high and low temperatures are the key questions in ecological and agronomical studies (Hua 2009).

Low temperatures limit the productivity and the geographical distribution of many important crops, through the negative impact that they exert in plant physiology. Cold stress can be classified as chilling (<20ºC) and freezing (<0ºC) stresses. Plants have developed a repertoire of adaptations to these conditions, such as seed and bud dormancy, vernalisation, photoperiod sensitivity and cold

acclimation (Penfield 2008). Cold acclimation is the process by which plants adquire freezing tolerance prior to the exposure to low non-freezing temperatures. This is followed by the remodelation of cell and tissue structures and reprogramming of metabolism and gene expression. Particularly, the responses to cold stress are characterised by profound modifications in metabolome and transcriptome (Chinnusamy *et al.* 2007). Cold stress induces the accumulation of a large amount of metabolite products (~75%), mostly osmolytes and other metabolites that function as signals for gene expression reconfiguration (Kaplan *et al.* 2004). Simultaneously, the cold-induced transcriptome is regulated by a complex transcriptional network (ICE/CBF pathway), important post-transcriptional (pre-mRNA splicing, export of mRNAs and small RNAs) and post-translational (ubiquitination /26S proteosome pathway and sumoylation) regulation processes (Chinnusamy *et al.* 2007).

Plant perception and response to high temperature/heat stress (HS) occurs when the rise in temperature, usually 5-7ºC, is above a threshold level (maximum temperature) (Wahid *et al.* 2007). Plants exhibit a complex response to extreme high temperatures in an attempt to survive and optimise growth and reprodutive success (Penfield 2008). The basal thermotolerance describes the plant response to HS in absence of any period of acclimatisation. On the other hand, acquired thermotolerance results from the prior exposure to a conditioning temperature, which is usually a short, sublethal HS or other moderate stress. The adquired thermotolerance is a more general mechanism that contributes to homeostasis of metabolome, transcriptome and proteome under diurnal temperature fluctuactions (Chinnusamy *et al.* 2007; Larkindale and Vierling 2008). When plants are exposed to low or high temperatures, several plant tissues and physiological processes are dramatically affected. The acquired temperature stress tolerance developed by plants in each particular temperature stress is distinct at physiological and molecular levels (Nagarajan and Nagarajan 2010).

1.3.1 Plant responses to heat stress

Plants can develop a broad range of morphological, physiological and molecular responses when exposed to HS (Figure 1.3). Plant responses to heat should be balanced to achieve optimal plant growth and productivity. In many cases, plant responses to a sudden increase of temperature (intensity) or long exposure (duration) may not be adequate, leading to plant death.

The HS immediately affects the photosynthetic apparatus, since the over-production of oxidative by-products induces the chlorophyll degradation and the disassembly of the photosystem II. As a consequence, the photochemical reactions and carbon metabolism are highly affected by HS

(Larkindale *et al.* 2007; Wahid *et al.* 2007). In addition to the typical sunburns in leaves and stems, the first impact of HS on plant development is the inhibition of shoot and root growth and early senescence of meristematic tissues, including internodes (Wahid *et al.* 2007). On the other hand, HS can also affect plant reproduction, including defects in the development of gametes, pollen germination and fertilisation.

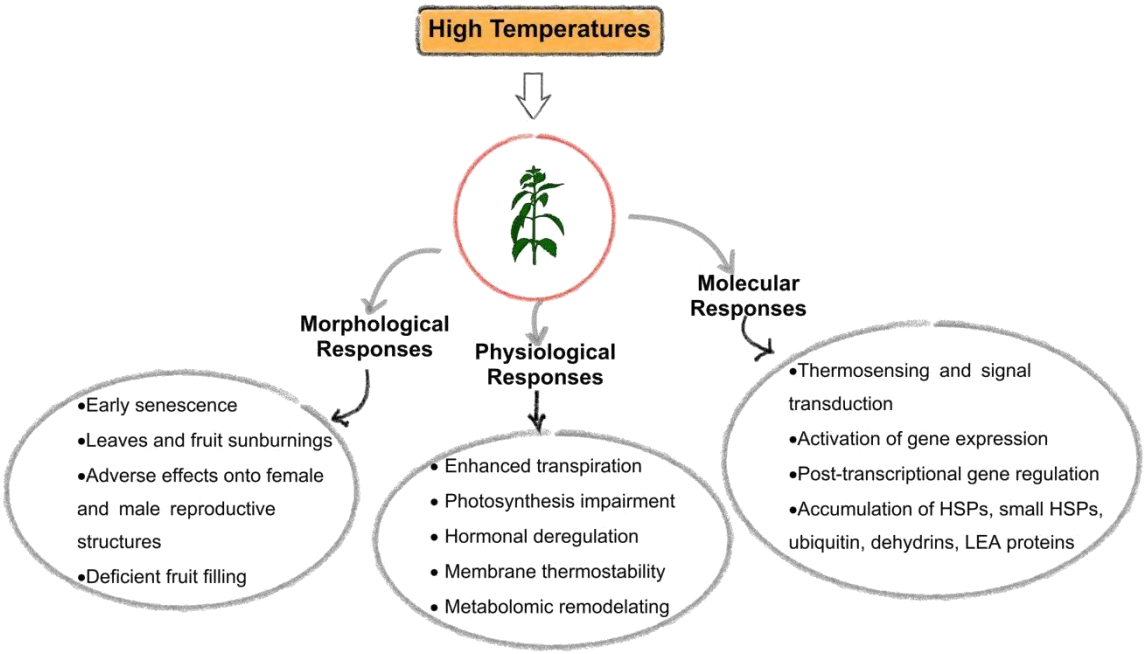


Figure 1.3. Overview of plant morphological, physiological and molecular responses induced by high temperatures. Under high temperatures, plants built a complex network of responses to HS. In addition to morphological modifications, one of the first targets of HS-induced damaging is the photosynthetic apparatus. At this level, other cellular structures are similarly affected (plasma membrane, endomembranes), as well as several metabolic pathways and hormonal homeostasis. Simultaneously, a complex and specific molecular response is built, in attempt to promote the thermotolerance development. Adapted from Wahid *et al.* (2007).

To respond to HS, plants adjust their metabolic, physiological and molecular processes. One of the first plant adaptation to HS is the accumulation of specific organic compounds, called osmolytes or compatible solutes. These compounds of low molecular mass promote the functional integrity of proteins and membranes. Recent metabolomic studies evidence that some amino acids (β-Ala and proline), sugars (maltose, sucrose and trehalose) and glycerol accumulated after prolonged exposure to HS (Kaplan 2004; Lv *et al.* 2011; Rizhsky *et al.* 2004). The hormonal homeostasis is also altered under HS, affecting the hormone levels of abscisic acid (ABA), salicylic acid (SA) and ethylene. These phytohormones regulate many physiological properties by acting as important signal molecules (Larkindale *et al.* 2007).

Plant responses to HS are mainly determined by key molecular modifications that occur at the cellular level. After HS perception on the plasma membrane, the signalling transduction of the signal

will promote changes at different levels of gene regulation: transcriptional, post-transcriptional, translational and post-translational. The transcriptional level of regulation includes the HS-specific induction of transcription factors (TFs), heat shock proteins and other stress-related proteins. Proteins related to RNA metabolism, signal transduction effectors, and post-translation modification (phosphorylation, sumoylation, methylation, ubiquitination) also perform specific and crucial regulation roles. All the molecular networks engaged in response to HS integrate crucial proteomic, metabolomic and transcriptomic modifications which are necessary for development of plant thermotolerance (Urano *et al.* 2010).

The negative effects of HS can be further intensified with the input of other stresses. For example, the combination of high temperatures and drought has been extensively studied, once they usually occur in the field simultaneously. This combination has a significantly greater detrimental effect on the growth and productivity of several crops, as well as unique physiological and molecular aspects (Mittler 2006).

1.3.2 Temperature perception and signalling transduction

Plants have a plethora of molecular processes to deal with HS, avoiding the negative effects caused by high temperatures. The activation of such molecular processes implicates several signalling pathways, which culminate in the activation of heat shock factors (HSFs) and the accumulation of high levels of heat shock proteins (HSPs) and small HSPs (sHSPs) (Figure 1.4). Meanwhile, the expression of other effectors components, such as dehydrins, late embryogenesis abundant (LEA) proteins, ROS-scavenging proteins also contributes for thermotolerance resistance.

Despite the intensive research on this area, the existence of a thermosensor has not been yet described in plants. Plasma membrane and associated Ca^{2+} channels are considered good candidates to be heat sensors in plants (Reddy *et al.* 2011; Saidi *et al.* 2011). Osmotic stress, cold and in particular HS can dramatically modify the activity and integrity of plasma membrane and its associated proteins (Falcone *et al.* 2004). Supporting this premise, it is likely that membrane fluidity during HS imposition affects the activity of specific proteins, namely Ca^{2+} channels. Accordingly, a specific and transient Ca^{2+} influx across the plasma membrane is triggered by heat, which promotes a sudden increase of cytoplasmic Ca^{2+} (Reddy *et al.* 2011). The Ca^{2+} mediated signal implicates other proteins that work as Ca^{2+} sensors, namely calmodulin (CaM), CaM-like proteins (CMLs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) (DeFalco *et al.* 2009; Reddy *et al.* 2011). Some of these Ca^{2+} sensors are localised in the nucleus, whereas others are translocated from the cytoplasm to the nucleus in response to stresses. The heat-induced increase

of cellular Ca^{2+} levels ultimately promotes changes in the expression of several HSFs and Ca^{2+} sensor-coding genes (Reddy *et al.* 2011). In addition, Ca^{2+} modulates the activity of HSFs through CaM-binding kinases and phosphatases.

Besides the Ca^{2+}, the HS signalling pathways include other secondary messengers, such as hydrogen peroxide (H_2O_2), D-myo-inositol-1,4,5-triphosphate (IP3) and phosphatidyl inositol 4,5-biphosphate (PIP2) (Figure 1.4). Hormones like ethylene and ABA are also implicated in the HS response, supporting the idea that heat is a major threat to plants, which developed redundant pathways to detect the stress caused by high temperatures. Recent evidences indicate that HS can cause some oxidative stress, depending on the duration of the stressful conditions. In the beginning of HS, the photosynthetic impairment originates high levels of reactive oxygen species (ROS), namely superoxide anion, which is immediately converted into H_2O_2. This ROS has been described as an essential second messenger in the HS signalling pathway, up-regulating *HSP* and *APX2* genes (Miller and Mitller 2006). This up-regulation appears to be mediated by HSFA2 and HSFA3 regulators (Miller and Mitller 2006; Suzuki and Mittler 2006).

Temperature variations can cause changes in membrane composition and fluidity (Mittler *et al.* 2012). Under HS, mobilisation of numerous lipids molecules that are known to be involved in signalling occurs, notably PIP2 and phosphatidic acid (PA). PA and PIP2 function as key mediators of signalling pathways, membrane dynamics and cytoskeleton organisation that occur between the cytoplasm and the nucleus (Mishkind *et al.* 2009). The PIP2 molecule is converted to IP3 by phospholipase D (PLD). Together with its derivate IP6, IP3 will be responsible for the release of Ca^{2+} from intracelular stores (Mishkind *et al.* 2009).

Besides the regulation of gene transcription, the secondary messengers mentioned above can also regulate protein activity. Together with a set of kinases and phosphatases, the secondary messengers promote the activation of transcription factors from the HSFA1 group. In plants growing in optimal temperatures, HSFA1 proteins are complexed and negatively regulated by the cytosolic HSP90s and HSP70s (Forreiter 2006). The accumulation of misfolded proteins during HS triggers the recruitment of HSP90/70 to repair protein damage and HSFA1s' activation is promoted. The HSFA1s' activation can also occur through phosphorylation performed by activated CaM-binding protein kinase (CBK), which is activated by MAP protein kinases, through H_2O_2 stimulation. Previous studies demonstrated that CaM-binding kinase 3 (AtCBK3) phosphorylates AtHSFA1a (Liu *et al.* 2008). This post-translational modification promotes HSFA1a conformational alteration (from monomeric to trimeric forms) and its binding to HSE elements of target genes, such as those coding for pivotal transcriptional factors, like HSFA2, DREB2A and HSF7a/7b (Figure 1.4). In turn, these factors promote the expression of a subset of HS-induced genes, building up a thermotolerance response (Liu *et al.* 2011).

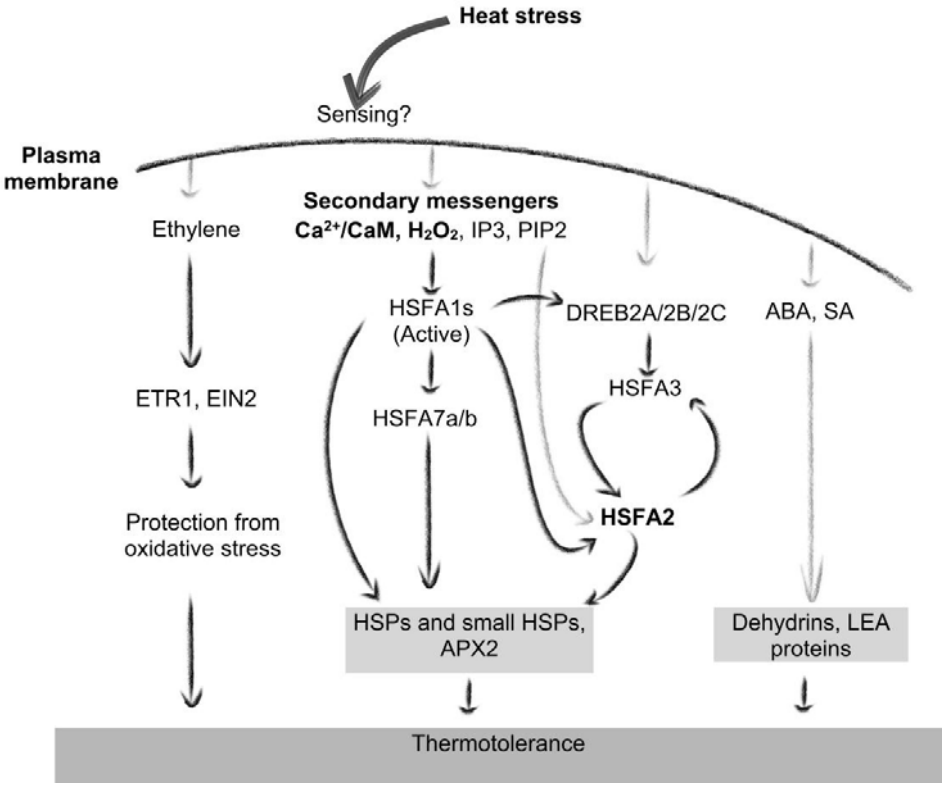


Figure 1.4 Overview of multiple signalling pathways and factors implicated in the HS response. HSFs are the main components of the network, mediating the expression of protective proteins - HSPs, small HSPs (sHSPs) and ascorbate peroxidase 2 (APX2). Some phytohormones such as ABA, SA and ethylene have also been implicated in response to HS. Bold arrows depict the signalling pathways already experimentally described, whereas the lighter ones are the only predicted. The secondary messengers Ca^{2+}/CaM and hydrogen peroxide (H_2O_2) (bold) have been described as early effectors of signalling pathways in response to HS. HSFA2 (bold) has been described as pivotal transcriptional regulator in late HS responses.

The HSFA2 transcription factor expression is highly expressed during HS, particularly in the later phases of the HS response (Schramm *et al.* 2006; Charng *et al.* 2007). However, HSFA2 role is not restricted to HS, since it is also implicated in other abiotic stresses (Nishizawa *et al.* 2006). Several results indicate that HSFA2 may be present in a signalling cascade under the control of at least one master regulator or directly induced by H_2O_2 (Li *et al.* 2005; Volkov *et al.* 2006).

DREB2A and its homologue DREB2B were initially indicated as transcriptional factors whose expression was induced under drought and high salinity (Sakuma *et al.* 2006a). However, recent data have also implicated DREB2A in high temperature responses (Schramm *et al.* 2008). Like HSFA1 members, DREB2A requires a phosphorylation to be activated under stressful conditions. Although DREB2A can be directly activated by HS, it also requires the HSFA1-inducible activation, in order to induce *HSFA3* expression (Figure 1.4). DREB2A involvement through HSFA3 allows the long-term imposition of HS responses (Schramm *et al.* 2008).

HSF7a/7b have been described as contributing to heat acclimation, in response to cytosolic unfolded protein accumulation during HS treatment (Larkindale and Vierling 2008; Sugio *et al.* 2009).

Globally, the activity of both early (HSFA1, DREB2A) and late (HSFA2, HSFA3 and HSF7a/7b) regulators of HS response leads to the expression of specific genes, responsible for thermotolerance acquisition in plants.

In addition to a plethora of transcription factors, HSPs and sHSPs are also highly expressed during sudden or gradual HS treatment (Huang and Xu 2008). However, these proteins are also engaged in other processes, namely embryogenesis, seed germination and pollen development. HSPs function as molecular chaperones, binding to structural unstable proteins. This role is important in protein folding, transport of proteins across membranes, modulation of protein activity and regulation of protein degradation. The molecular roles of HSPs are consistent with their wide subcellular distribution (Table 1.2). Hence, their intervention in diverse development stages appears to be essential for proper functioning of cell, in particular maintenance of cellular homeostasis (Forreiter 2006).

There are five well-characterised classes of HSPs that have been defined in both plants and other organisms (Wang *et al.* 2004b). As HSP classes are common to all organisms and their function was preserved during evolution (Forreiter 2006; Tiedemann *et al.* 2008), the prokaryotic counterpart of a given chaperone is also presented in table 1.2 (in brackets).

HSP100/Clp class of chaperones is found in many organisms (bacteria, yeast, plants), belonging to AAA+ superfamily of ATPases (Singh and Grover 2010). In plants, they are considered as caseinolytic protease (Clp)-like proteins, working to maintain the quality of cellular proteins. These proteins are structurally hexameric and their ATP-dependent activity promotes the protein remodelling through ATP-binding and hydrolysis (Singh and Grover 2010). In Arabidopsis, the well characterised AtHSP101 has been implicated in acquired thermotolerance in different growth stages (Larkindale *et al.* 2007).

HSP90 chaperones are well characterised in a number of eukaryotes (mostly in animals and yeast). In Arabidopsis, there are seven *HSP90* genes, whose proteins have different subcellular localisations. Although some of these genes show high expression levels in response to heat treatment, no direct evidences of connection between HSP90s and heat tolerance have been described (Forreiter 2006). Their activity may depend on the type of interacting partner involved (receptor/signalling molecule) but an interaction with HSP70 and co-chaperones is necessary (Forreiter 2006).

Table 1.2 Principal groups of HSPs in plants. HSP roles and respective subcellular localisations show their ubiquity and partial functional overlapping in the cell.

HSP class	Size (kDa)	Examples	Cellular localisation	Principal functions
HSP100 (Clp)	100-114	AtHSP101	Cytoplasm, mitochondria and chloroplast	Resolubilisation of protein aggregation in cooperation with HSP70/DnaK chaperone system and sHSPs. (Weibezahn *et al.* 2004; Singh and Grover 2010). AAA+ ATP-dependent activity (Neuwald *et al.* 1999).
HSP90 (HtpG)	80- 94	AtHSP90	Cytoplasm, mitochondria, ER and chloroplast	Folding of proteins with key functions in cell proliferation (Johannes 1999). Protein trafficking and degradation (Wang *et al.* 2004b). Roles in signalling transduction pathways (Stepanova *et al.* 1996; Johannes 1999). Activity promoted by binding of HSF70 and co-chaperones (Forreiter 2006).
HSP70 (DnaK)	69- 71	AtHSP70, AtHSC70	Cytoplasm, nucleus, mitochondria and chloroplast	Plant heat tolerance (Nover and Scharf 1997). Necessary for activity of HSP90, HSP101 and sHSPs (Young *et al.* 2004). ATP binding and hydrolysis (Forreiter 2006).
HSP60 (GroE)	57-60	AtTCP-1	Mitochondria and chloroplast	Assistance in folding of new synthesised and new translocated proteins to achieve their native forms (Frydman 2001)
Small HSPs, sHSPs	12-40	AtHSP17.7 AtHSP18.1 AtHSP25.3 AtHSP23.6 AtHSP26.5 HSA32	Cytoplasm, nucleus, plastids, ER, mitochondria	Prevention of irreversible aggregation of unfolding proteins (Forreiter 2006). Association with membranes and maintenance of integrity (Nover *et al.* 1989; Friedrich *et al.* 2004). ATP-independent activity (Huang and Xu 2008).

HSP70 proteins play a pivotal role in controlling HSP90, HSP101 and sHSPs activity, adjusting the global chaperone system activity. Due to its weak ATPase activity, HSP70 interacts with HSP40 to increase its activity (Bukau and Horwich 1998). Several studies in plants have demonstrated that HSP70s and their related isoforms are important for plant heat tolerance (Larkindale *et al.* 2007).

Less known than other HSPs' groups, plant HSP60s or chaperonins, have been described as a chaperone system similar in structure and function to the procaryotic GroEL/S complex (Forreiter 2006). They exist as two distinct groups, being associated to organelles (group I) or distributed in cytosol (group II). Both groups of chaperonins play roles in assistance to newly synthesised and newly translocated proteins (Huang and Xo 2008). While chaperonins in organelles function in complex with other chaperones, some cytosolic chaperonins join with HSP70/HSP40 complex and assist in actin protein folding mechanisms (Frydman 2001).

Among the five major conserved groups of HSPs/chaperones, the sHSPs are found in all kingdoms. They are the most prevalent group in plants and are present in diverse cellular compartments (Larkindale *et al.* 2007). They bind to a wide range of cellular substrates and are implicated in many different stresses. These proteins possess an oligomeric organisation, which is broken down into smaller dimers under stressful conditions (Forreiter 2006). When sHSPs-substrates complexes interact with other molecular chaperones they can be stabilised and promote refolding. A high sHSPs/substrate ratio is the principal determinant in co-interaction and activity efficiency of sHSPs with unfolded proteins (Nakamoto and Vígh 2007; Siddique *et al.* 2008).

HS responses also involve modifications in hormonal levels. ABA is known to be involved in HS response, inducing some degree of thermotolerance in plants (Rock *et al.* 2010). In addition to up-regulation of ABA biosynthesis genes (*ABA1, NCED2, NCED5* and *NCED9*) and enhancement of *SPINDLY* gene expression (*SPY*, a GA negative regulator), high ABA levels also promote the accumulation of HSPs, dehydrins and LEA proteins. Despite these evidences, ABA was suggested to function in the HS response through a HSF/HSP-independent pathway (Larkindale *et al.* 2007). Other experimental evidences suggest that ABA functions in preventing denaturation and coagulation of cellular proteins or membranes under HS (Rock *et al.* 2010).

SA hormone is also accumulated under HS conditions and improves heat tolerance. SA was suggested to stabilise the trimers of HSFs and help them to bind to HSE sequences of HS-related promoters (Larkindale *et al.* 2007).

The present knowledge about the ethylene involvement in thermotolerance is still scarce. Previous studies indicated that ACC oxidases (ethylene biosynthesis), as well as *ETR1* and *EIN2* genes are up-regulated during heat treatment (Larkindale *et al.* 2007). Ultimately, they seem to protect against the oxidative stress generated by high temperatures.

In synthesis, HS responses are supported by a complex network between all the cellular components above described. This network promotes plant thermotolerance improvement and adaptation to new environmental conditions.

1.3.3 Genetic improvement for heat tolerance

When plants are subjected to different environmental conditions, a high number of genes is up- or down-regulated, resulting in changes of several metabolites and proteins levels. Ultimately, these changes on metabolites and proteins are the principal factors that confer plant protection against the imposed stresses.

Plant scientists have been concerned in manipulating the molecular processes used by plants in response to abiotic stresses. The main goal is to improve crop growth in adverse conditions (Bhatnagar-Mathur *et al.* 2008). The introduction of molecular markers use in breeding programs, together with introgression of genomic portions (QTLs), have permitted the selection of better agronomic characteristics. However, the lack of a precise knowledge of key genes underlying the QTLs leads to the development of genetically engineered or transgenic plants (Bhatnagar-Mathur *et al.* 2008). Transgenic plants are defined by the introduction and/or over-expression of specific genes in the plant. In addition to be a faster way to insert beneficial genes, this genetic engineering approach is the only option when genes of interest come from cross barrier species, distant relatives, or from non-plant organisms (Bhatnagar-Mathur *et al.* 2008).

Various transgenic technologies have been used to improve stress tolerance in plants, in particular by the introduction of components involved in HS response (Bhatnagar-Mathur *et al.* 2008). Although many molecular mechanisms of HS response maintain to be elucidated, the gene expression profile during HS is one of the best studied inside abiotic stresses. It is known that responses to HS are characterised by synthesis of HSPs, whose expression is regulated by HSFs. The growing need to obtain HS tolerant crops through transgenic approaches lead to the manipulation of some HS response components, namely HSFs and HSPs accumulation (directly or through regulatory circuits governed by HSFs), changes in levels of osmolytes and ROS-scavenging enzymes (Bhatnagar-Mathur *et al.* 2008; Singh and Grover 2008).

Since HSPs are involved in thermotolerance acquisition in plants, their up-regulation was achieved in several studies. Malik *et al.* (1999) produced transgenic carrot cell lines and plants over-expressing *sHSP17.7*. Modified expression of *sHSP17.7* enhanced carrot survival at high temperature. Transgenic rice plants over-expressing *OsHSP17.7* gene also showed increased thermotolerance as well as higher resistance to UV-B radiation (Murakami *et al.* 2004). The over-expression of *HSP101* in rice produced plants with high survival rates in the post-HS recovery phase (Katiyar-Agarwal *et al.* 2003). The over-expression of At*HSFA3* and tomato *HSFA1* genes lead to enhancement of thermotolerance of respective transgenic plants (Prändl *et al.* 1998; Mishra *et al.* 2002).

Plant response to HS also involves the synthesis of specific osmolytes, such as proline, glycine-betaine and sugars (mannitol, trehalose, sorbitol). Osmolytes accumulation helps plants to adapt against water deficit generated by continuous exposition to HS (Larkindale *et al.* 2007). Plants harbouring transgenes encoding enzymes implicated in the biosynthesis of specific osmolytes have been produced and the consequences of osmolytes accumulation addressed. The most extensive study promoted the accumulation of glycine-betaine in Arabidopsis through the production of plants over-expressing the *codA* gene (encodes for choline oxidase from *Arthrobacter globiformis*) (Alia *et al.* 1998). The seeds of such transgenic plants were more resistant to HS than the wild-type seeds, resulting in a higher rate of seed germination and increased growth of seedlings. Recently, the over-expression of betaine aldehyde dehydrogenase protein from spinach increased the glycine-betaine levels in tobacco plants (Yang *et al.* 2005). The transformant seedlings showed increased thermotolerance as well as higher CO_2 assimilation rate.

The ROS characteristic accumulation of HS is reduced by scavenging enzymes, such as ascorbate peroxidase (APX). The over-expression of barley *HvAPX1* gene in Arabidopsis increased their thermotolerance, when compared to wild-type (Shi *et al.* 2001).

Since the manipulation of gene expression levels normally implies many molecular and physiological modifications, the “omics” (genomic, transcriptomic and proteomic) studies are essential to characterise the key components involved at different regulation levels (namely post-transcriptional and post-translational).

1.4 RNA-binding proteins, crucial effectors in post-transcriptional regulation

Gene regulation can occur at transcriptional, post-transcriptional, translational and post-translational levels. However, the study of gene regulation during biotic and abiotic stresses is mostly focused on the transcriptional level. Only recently the other levels of gene regulation have started to be thoroughly analysed. As a result, the importance of post-transcriptional, translational and post-translational regulation in stress signalling and molecular responses is still far from being elucidated. These levels of regulation have risen as key mechanisms to modulate the amount and activity of transcripts and proteins under stressful conditions (Urano *et al.* 2010).

The amount of mRNAs available in the cell for translation can be controlled through different steps, which include transcription, mRNA processing, transport, translation initiation and mRNA turnover. All these processes implicate direct and/or indirect binding of proteins to RNA molecules

(Glisovic *et al.* 2008). These proteins are designated RNA-binding proteins (RBPs) and compose a widespread family.

The binding of proteins to mRNA in the nucleus during gene transcription and RNA processing forms the heterogeneous nuclear ribonucleoproteins (hnRNPs) (Chaudhury *et al.* 2010). Molecular cloning of genes encoding hnRNPs led to the discovery of several motifs/domains involved in RNA binding and protein-protein interactions (Burd and Dreyfuss 1994). Some well characterised RNA-binding domains in ribonucleoproteins include: RNA-recognition motif (RRM); K-homology (KH) domain; RGG box (Arg-Gly-Gly); DEAD/DEAH box; zinc finger (ZnF); double stranded RNA-binding domain (dsRBD); Pumilio/FBF (PUF) domain and Piwi/Argonaute/Zwille (PAZ) domain (Chen and Varani 2005; Lunde *et al.* 2007; Glisovic *et al.* 2008). Using bioinformatics tools, a high number of RBPs with different combinations of RNA-binding domains was discovered, in eukaryotic organisms.

In Arabidopsis, a high number of RBPs (279) were recently detected, compared to the 100 RBPs identified in *Caenorhabditis elegans* and 117 RBPs in *Drosophila* (Peal *et al.* 2011). Arabidopsis RBPs mainly contain RRM domains and others RNA-binding domains that have not been described yet. Recent evidences indicate that besides RNA recognition and binding, the RRM domain is also implicated in protein-protein interactions (Maris *et al.* 2005). This may be important in the establishment of a broad range of protein associations that are necessary to modulate the RNA-binding affinity and specificity. The RRM domain has approximately 90 amino acids and contains the RNP1 and RNP2 consensus sequences or motifs. The RNP1 is a central and highly conserved sequence, containing eight conserved residues that are mainly aromatic and positively charged. This motif has been indicated to be responsible for the RNA interaction. The RNP2 possesses six amino acids and is less conserved than RNP1 (Lorković and Barta 2002) .

In addition to RRM domain, the RBPs can harbour other functional domains that are mostly involved in protein-protein interactions and post-translational modifications (Lorković and Barta 2002; Peal *et al.* 2011). In Arabidopsis, these domains include glycine(G)-rich, arginine-(R)rich, serine/arginine(SR)-rich, glutamine(Q)-rich and poly(A)-binding (KRDE) domain (Lorković and Barta 2002; Zdravko 2009).

The Arabidopsis RRM-containing proteins are divided in groups, based on similarities with their metazoan counterparts and on the combination between RRM and the functional domains involved in protein-protein interaction domains. Four main groups can be distinguished: the poly(A)-binding proteins (PABPs); SR proteins (including snRNPs); oligourydilate-binding proteins and G-rich-RBPs (GR-RBPs) (Figure 1.5).

The PABPs are composed of four consecutive RRMs and may have an additional functional domain. These proteins bind to poly(A) tails of mRNAs, being essential for polyadenylation

stimulation, control of poly(A) length, regulation of mRNA stability, translation initiation and for mRNA degradation (Keller and Minvielle-Sebastia 1997; Minvielle-Sebastia and Keller 1999; Wahle and Rüegsegger 1999). The Arabidopsis genome codifies for 12 different PABPs, but nine of them are homologues to yeast and mammalian Pab1p (Lorković and Barta 2002).

The SR proteins, together with snRNPs, are the major effectors in mRNA splicing activity and spliceosome composition. SR proteins play an important role in canonical and alternative splicing by promoting interactions across intronic and exonic sequences during early steps of the spliceosome assembly (Duque 2011). Together with snRNPs (U1, U2, U4/U6 and U5), the SR proteins are important for selection of specific sequences (branchpoint, 5' and 3' splicing sites) during the pre-mRNA splicing (Barta *et al.* 2008).

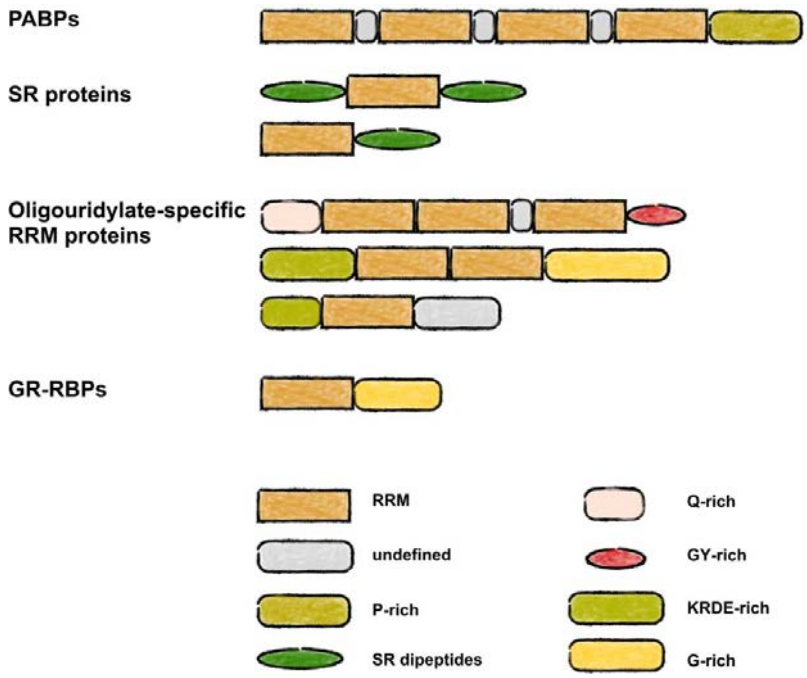


Figure 1.5. Modular structure of the Arabidopsis RRM-containing proteins. Only representative types of domain combinations are shown. Individual domains are identified by different shapes and colours. Different types of domains (RNA-binding and functional/auxiliary domains) are listed at the bottom. Adapted from Lorković and Barta (2002).

Oligourydilate-binding proteins include UBP1 (and related proteins UBA1 and UBA2), RBP45 and RBP47. Despite their specificity in mRNA stability and pre-mRNA splicing, these proteins are structurally similar, with three RRM domains (except UBA1 and UBA2) (Peal *et al.* 2011). The RBP45/RBP47 and UBP1 proteins are homologous to yeast Nam8p and metazoan TIA-1 (Lorković and Barta 2002). Arabidopsis GR-RBPs harbour RRMs at the N-terminus and glycine-rich region at the C-terminus (Lorković and Barta 2002). GR-RBPs have been described to be active during development, response to stimulus, such as circadian clock and several environmental stresses (salt, cold).

In summary, plants possess a large number of RNA-binding proteins, crucial for several post-transcriptional mechanisms that control gene expression. This RBP diversity is partially responsible for the success of plant adaptation during evolution. Although the function of the majority of RRM-containing proteins still remains unknown, the progressive technical advances in transcriptome and proteome analyses will be essential to elucidate most of their functions under different environmental conditions.

1.4.1 Alternative splicing, the key for proteome diversity

During RNA splicing, introns are removed from primary transcripts and the exons are joined to form a continuous sequence that specifies a functional polypeptide. This process is performed by a large ribonucleoprotein complex – spliceosome – composed by snRNPs, SR proteins and other splicing regulators (hnRNPs, SR kinase proteins) (Kim *et al.* 2008a). Just after the emerging of pre-mRNA, several spliceosome complexes are assembled along specific sequences present in the pre-mRNA molecule. These sequences define exon-intron the boundaries. The main splice recognition sites in plants are: 5' donor (AG/GUAAG) and 3' acceptor (UGCAG/G) splice sites and branchpoint (Figure 1.6).

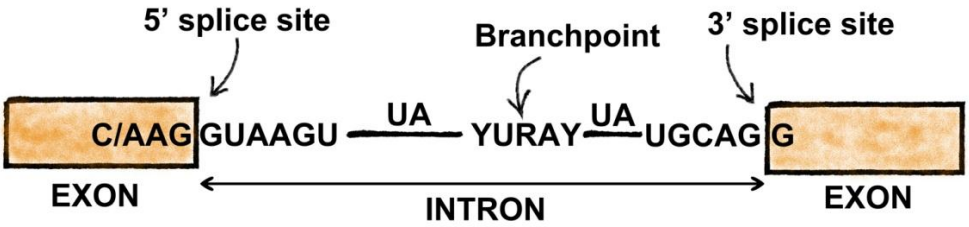


Figure 1.6 Major consensus sequences involved in the splicing process in plants. The three principal splicing signals are depicted: 5' splice site, branchpoint and 3' splice site. The UA corresponds to UA-rich intronic sequences. Adapted from Brown (1996).

The complexity of RNA splicing is firstly determined by both 5' and 3' splice recognition sites. Although many sequences similar to the consensus might be present in the pre-mRNA, the existence of a branchpoint (AU rich) and a U-rich polypyrimidine sequences inside the introns are crucial for the selection of the correct splice sites. The absence of these two elements leads to the wrong selection of splice sites (Brown 1996; Kim *et al.* 2008).

The efficiency of RNA splicing is enhanced by short *cis*-acting regulatory sequences (4-18 nts) that are classified as exonic or intronic splicing enhancers or silencers. Specific binding of splicing regulator proteins, such SR proteins, snRNPs and hnRNPs to those *cis*-acting elements assists in the correct position of the spliceosome on splice sites.

Depending on the environmental or developmental inputs, the splicing pattern could be modified by the recognition of a new splice site, leading to alternative splicing (AS). This process promotes the generation of more than one mRNA transcript from the same pre-mRNA (Brown 1996; Reddy 2007; Kim *et al.* 2008). AS plays an important role in increasing the protein diversity, an essential aspect to maintain the complexity of an organism. In plants, the AS is precisely regulated in a tissue- and developmental stage-specific manner, encompassing the majority of genes related to cell growth and maintenance, cell communication and plant development (Barta *et al.* 2008). In plants and other organisms, the major AS events can result from the selection of alternative 5' or 3' splicing sites, retention of an intron or skipping of exons (Figure 1.7) (Barbazuk *et al.* 2008)

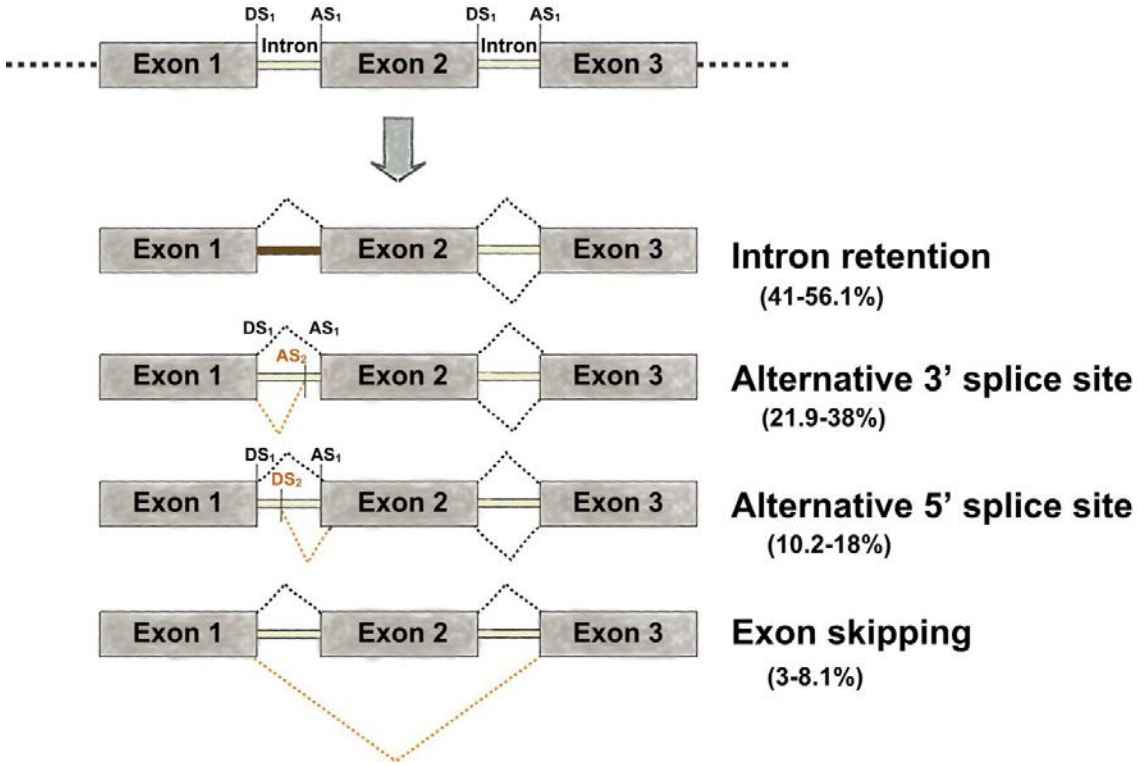


Figure 1.7 The main types of alternative splicing in plants. Four different alternative splicing events can be distinguished in plants: intron retention, alternative 3' splice site selection, alternative 5' splice site selection and exon skipping. The relative prevalence of each type of alternative splicing in Arabidopsis is shown in parenthesis. Dashed lines indicate the splicing options: canonical or constitutive splicing (in black, above); and alternative splicing (in orange, below). The brown intron corresponds to a retained intron, resulting from intron retention mechanism. DS1, donor or 5' constitutive splice site; DS2, donor or 5' alternative splice site; AS1, acceptor or 3' constitutive splice site; AS2, acceptor or 3' alternative splice site. Adapted from Ner-Gaon *et al.* (2004) and Barbazuk *et al.* (2008)

Intron retention has been reported as the major AS event in plants, occurring in more than a half of the alternative splicing events in Arabidopsis. This mechanism results in the introduction of a few amino acids in the final protein sequence, as a result of retention of the entire intronic sequence. The effect of a retained intron strongly depends from its localisation in mRNA transcript. The retained intron could appear either as a part of coding sequence (CDS), bridging both CDS and UTRs or be

present in 5'/3' UTR (Ner-Gaon *et al.* 2004; Barbazuk *et al*, 2008).These different possibilities of intron retention can have profound effects on gene expression. When retained in CDS, it may result in different effects, depending on the tissue and developmental stage. Normally, this mechanism produces a shorter protein that prematurely ends at a small distance from 5' end of retained intron (Ner-Gaon *et al.* 2004; Barbazuk *et al.* 2008). However, the majority of transcripts that harbour an in-frame premature stop codon (PTC) are good candidates for further degradation. In case of an intron retention in the 5'UTR, the tissue specificity, expression levels and translation efficiency of alternative transcripts could be altered (Gauss *et al.* 2006). The intron retention into 3'UTR may have drastic effects on mRNA stability (Chan and Yu 1998; Cheng *et al.* 1999).

Another very common and relevant type of AS in plants is the alternative acceptor or donor splice sites (or alternative 3' or 5' splice sites, respectively). Depending on whether an alternative 3' or 5' splice site is used, either the 3'-most or 5'-most exon is extended, provided the splice does not change the reading frame. However, if the reading frame is changed the generation of an in-frame stop codon frequently occurs, leading to a truncated protein product (Louzada 2007).

In plants, in contrast to humans, the exon skipping (splicing or inclusion of an exon) is a less common form of AS. In this mechanism, an exon is either included or excluded from the mRNA (Louzada 2007; Barbazuk *et al.* 2008).

1.4.1.1 Regulation of alternative splicing under stress

Research on gene expression regulation at transcriptional level has resulted on the identification of stress response-related transcription factors and key signalling components. However, many studies have revealed that AS events occur on stress-related transcripts under abiotic stresses (Ali and Reddy 2008a). Indeed, AS has been considered a major gene regulation process in stress responses, since the resulting products generate great transcriptome/proteome alterations important for stress adaptation (Ali and Reddy 2008a).

During adaptation to extreme temperatures and after the induction of HS-related genes, AS has been detected in several *SR* transcripts (*SR30, SR33*, *SCL30a, RS31, SR34b*) (Palusa *et al.* 2007). The resulting isoforms act in combination to specifically alter the splicing process of downstream temperature-induced genes. For example, in some plant crops, specific members of ERF/AP2 family transcription factors are predicted to undergo AS in cold conditions (Iida *et al.* 2005). On the other hand, several studies have shown changes in the AS pattern of *HSP* transcripts (*HSP70, HSP81*) (Hopf *et al.* 1992; Larkin and Park 1999). The changes in alternative splicing apparently would lead to either an enhancement or reduction of the HSP activity.

The changes in AS regulation can greatly depend from a combination of several *cis* and *trans* splicing elements which appear to be crucial for AS regulation (Ali and Reddy 2008a). Nevertheless, the *cis* elements that respond to temperature stress are mostly unknown. The *trans* activity is mostly addressed by SR proteins in combination with other splicing factors, which can act as splicing enhancers or repressors. In addition, the phosphorylation state of SR proteins could be determinant for the proper localisation and activity regulation of the several splicing components (van Bentem *et al.* 2006). The biophysical conditions imposed by temperature stress can promote conformational rearrangements of RNA *cis*-splicing elements or modulation of thermal-dependent stability of protein-protein, RNA-RNA or RNA-proteins interactions. In the same way, the transcription rate influences the AS, once the sudden increase of transcription could lead to an exon or intron being skipped, resulting in unproductive spliced variants (de la Mata *et al.* 2003; Ali and Reddy 2008a).

1.4.2 mRNA Degradation Pathways: an Overview

The homeostasis of cellular transcriptome is mostly regulated under the flux of synthesis and degradation of RNA molecules. However, the kinetics established between these two mechanisms are greatly dependent on the RNA stability and RNA-associated proteins [forming a ribonucleoprotein (RNP) complex] (Bailey-Serres *et al.* 2009). Although the majority of studies related to mRNA turnover mechanisms have been performed in animals, recent advances have emerged in plants. Until now, the best way to characterise mRNA decay consisted in inhibiting the transcription, using chemical agents as actinomycin or cordycepin (Belostotsky 2008; Hori and Watanabe 2008). However, these chemical treatments can also lead to the depletion of some specific sets of genes encoding regulatory factors and effectors of mRNA stability.

Eukaryotic mRNAs are thought to undergo degradation through a defined sequence of steps that first require deadenylation at 3' terminus. After removal of adenines, two main degradation pathways pathways are present: deadenylation-dependent deccaping and deadenylation-dependent exosome (Figure 1.8) (Belostotsky 2008). Both mechanisms act in steady-state conditions and do not imply the existence of structural defects. In deadenylation-dependent decapping, the transcript is subjected to a decapping process (removing of m^7GDP cap in the 5' terminus). This process is played by specific deadenylating proteins, which can include DCP2 in combination with DCP1, DCP5 and VARICOSE (Xu and Chua 2011). After cap removal the access for 5'-3' exoribonucleolytic enzymes, such as homologous components of XRN1 family, is facilitated enhancing mRNA

degradation (Souret *et al.* 2004). Alternatively, the deadenylated mRNAs can be directed for degradation via the exosomal pathway, processed by 3'-5' exoribonucleolytic enzymes.

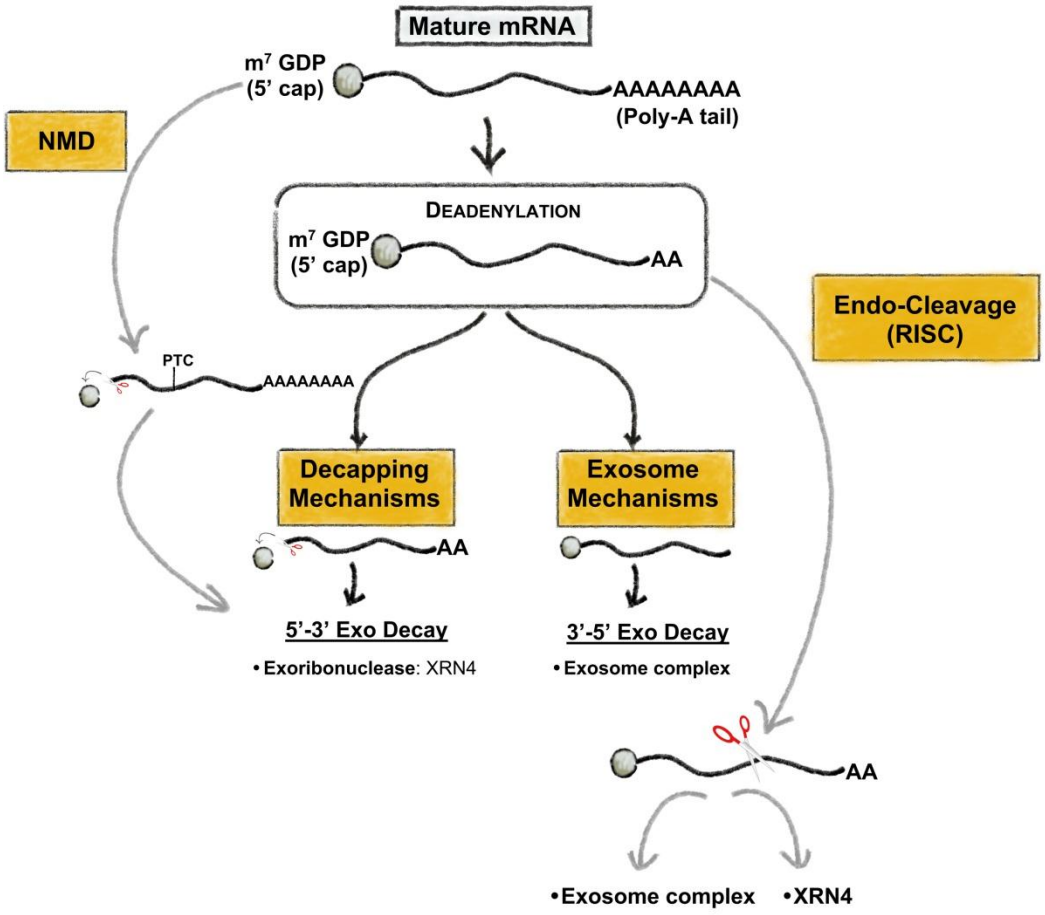


Figure 1.8 Principal mRNA turnover and decay pathways, with emphasis of some mRNA decay factors in plants. The main pathways for mRNAs structurally unaffected are deadenylation-dependent, either through decapping or exosomal mechanisms (bold arrows). Alternatively, two pathways of mRNA turnover and decay (light arrows) can occur: NMD (direct decapping) and endonucleolytic mRNA cleavage by RISC complex (normally miRNA and siRNA-programmed) followed by exosomal and XRN4-mediated decay. Adapted from Belostotsky (2008).

The exosomal pathway is executed through the action of exosome complex. The exosome consists in nine conserved subunits forming the core complex, which associates with active ribonucleases, RNA-binding proteins, helicases and additional co-factors (Lange and Gagliardi 2011). The eukaryote exosome core complex comprises three heterodimers that form a ring-like structure (RRP41-RRP45, RRP42-MTR3 and RRP43-RRP46 Rnase PH domain-type), to which a "cap" of three S1/KH domain proteins (RRP4, RRP40 and CSL4) are bound. Homologues of all nine core proteins are coded in plants (Lange and Gagliardi 2011). In interaction with auxiliary factors, the exosome 3'-5' exonucleotidic activities are executed in the cytoplasm (homeostatic mRNA turnover, decay of unstable mRNAs, NMD, products from RISC activity, no-go decay) and in the nucleus (3'

end processing of the 5.8S rRNA precursor, degradation of aberrant pre-rRNAs, pre-mRNAs and pre-tRNAs) (Chekanova *et al.* 2007).

Two pathways for mRNA decay can be recognised. Both are dependent on extrinsic and/or intrinsic stimulus and the existence of important structural signals. The mRNA decay can be started by internal endonucleolytic enzymes, mostly present in the RISC multi-complex, which cut the RNA sequence. This complex mediates the post-transcriptional gene silencing, and contains AGO proteins and single stranded small RNAs (siRNA or miRNA) (Jones-Rhoades *et al.* 2006). The resulting fragments can be degraded either via the exosome or the exonucleolytic enzyme XRN4 (Figure 1.8). When aberrant mRNAs containing PTC are detected the nonsense-mediated mRNA decay (NMD) eliminates these transcripts, avoiding their accumulation and further cellular toxicity. It has been suggested that aberrant transcripts are subjected to direct decapping and are degraded via 5'-3' exonucleotidic decay by XRN4 (Figure 1.8).

Studies performed in yeast and mammalian models demonstrated that RNA decay reactions are spatially compartmentalised. In plants, many enzymes and the exosome complex involved in RNA decay were suggested to be localised in small and discrete cytoplasmic structures. These structures, called processing bodies (P-bodies or PBs), are physical structures that establish the widespread cross-talk between the different processes of mRNA decay and translational control. In addition, they are strongly implicated in RNA interference processes (Parker and Sheth 2007).

1.4.2.1 Nonsense-mediated mRNA decay (NMD), an update mechanism for plant mRNA homeostasis

The NMD is one of several mechanisms involved in RNA surveillance pathways that ensure the fidelity of gene expression by degrading mRNAs that lack the proper arrangement of translational signals. This phenomenon is observed in all investigated organisms, from bacteria to mammalian cells, but has been extensively studied in eukaryotic cells (Brogna and Wen 2009). Although widely studied in animal models, the NMD process in plants still needs to be investigated in more detail.

The central question in NMD concerns how the process distinguishes between a PTC and a normal stop codon. Two NMD models have been proposed: the *faux* UTR model (*S. cerevisiae*) and EJC-based NMD model (mammalian cells) (Kerenyi *et al.* 2008; Brogna and Wen 2009).

The *faux* (false) model predicts that the distance between the PTC and the poly(A) tail might be the key determinant. The translation termination of PTC-containing mRNAs is suggested to be aberrant because their 3'UTR factors, including poly(A)-binding protein, are not properly positioned and

cannot interact with terminating ribosome (Amrani *et al.* 2004). If a long 3'UTR inhibits this interaction, the translation termination will be detected as aberrant and transcript is driven to degradation.

The other NMD model is based on exon junction complex (EJC)-interacting proteins. EJC is a multiprotein complex with a core of four proteins that interact with UPF2 (UP-frameshift 2) and UPF3 (Amrani *et al.* 2006). These components further interact with phosphorylated UPF1 to induce NMD mechanism. The Arabidopsis genome codifies for the three UPF homologues, but only UPF1 and UPF3 have been described (Hori and Watanabe 2005). The functional studies performed in Arabidopsis demonstrated that *upf1* and *upf3* mutants accumulate high levels of alternatively spliced mRNAs containing PTC. In addition, these mutants are extremely affected in some developmental stages, and display some lethality (Hori and Watanabe 2005; Yoine *et al.* 2006). During the pre-mRNA splicing, EJCs are deposited 20-25 nts upstream of each exon-exon junction. Simultaneously, the nuclear cap binding complex (CBC, comprising the binding proteins CBP80 and CBP20) is added to 5' cap of the pre-mRNAs (Lewis and Izaurflde 1997). During the nuclear export of mature mRNA, a ribosome or ribosomal subunit (usually 40S) binds and scan mRNAs for PTCs (named as '*pionner round*' process), displaying EJCs upstream of the stop codon (Maquat 2004; Chang *et al.* 2007; Brogna and Wen 2009). In earliest round(s) of translation, if the CBC is not replaced by initiation translation factors (eIF4s) and ribosome is prematurely terminated at PTC, occurs the formation of NMD-inducing complex, by recruiting of phosphorylated UPF1. As in PTC-containing transcripts there are at least one EJC deposited downstream of the PTC (>50-55 nts), phosphorylated UPF1 interacts with UPF2/UPF3 complex in EJC and move aberrant transcripts to degradation (Chang *et al.* 2007).

The mechanisms involved in plant NMD are still unclear. The detection of PTC and distinction from a normal termination codon in plants has been proposed to be in part similar to yeast and mammalian models. In plants, the PTC is suggested to be detected when the premature translation termination event takes place far upstream of the original 3'UTR (Hoof and Green 2006). The existence of a EJC > 50 nts downstream of this PTC avoids the interaction of terminating ribosome with specific sequences present in 3'UTR, which are essential for correct translation termination. Under this conditions, EJC recruits and activates UPF factors, priming the elimination of aberrant transcripts by NMD (Hoof and Green 2006; Brogna and Wen 2009).

Although little knowledge about plant NMD has emerged, the advances in transcriptome methodologies will be crucial to understand this and other mRNA-surveillance mechanisms during transition of non-stress to HS-induced transcriptomes in plants. The better comprehension of importance of these surveillance mechanisms in plant physiology will promote the prediction of tolerance of transgenic crop plants under episodic extreme temperatures in the field.

1.5 Genetic control of seed development and germination

Plants developed structures to promote their successful adaptation to environmental conditions. For example, in case of gimnosperms and angiosperms, the capacity for seed production allowed their evolutionary success. Seeds are structures originated from the double fertilisation of egg cell and the large central cell (polar nuclei). After development, they include the embryo and endosperm, respectively. During seed formation a set of developmental processes occur until seeds reach a quiescent state. At this stage the seed becomes dormant which is essential to turn it competent to germination. Seed development is tightly regulated by genetic processes, most of them controlled by hormonal homeostasis between ABA and GA hormones.

1.5.1 Molecular and physiological traits of seed development

The seed development processes are largely divided into three phases: embryo morphogenesis, embryo maturation and seed desiccation (Bentsink and Koornneef 2008) (Figure 1.9). During morphogenesis, cell division is very active and embryo undergoes through several developmental stages: pre-globular, globular and heart stages.

Following this early phase, the growth stops and developing seeds enter into a maturation phase. The metabolism undergoes reorganisation with intensive synthesis of storage compounds (starch, oil and storage proteins) and nucleic acids. The embryo accumulates considerable levels of ABA during this maturation phase, which can be physiologically divided in two phases (Figure 1.9): mid maturation (MEM) and later maturation stages (LEM). Besides the progressive accumulation of ABA, the seed also stores protective proteins such as LEA proteins, entering into a desiccation stage (Wise and Tunnacliffe 2004). After the desiccation process, the embryo enters into a dormancy phase (dormant seed), until favourable conditions allow seed germination (Vicente-Carbajosa and Carbonero 2005; Yamaguchi and Nambara 2007).

Seed development is regulated by temporal and spatial expression of stage-specific genes and is dependent on hormonal levels. Experimental data shows an up-regulation of a specific set of genes, mostly involved in gene transcriptional regulation, signalling and metabolic pathways (e.g. lipids and carbohydrates synthesis).

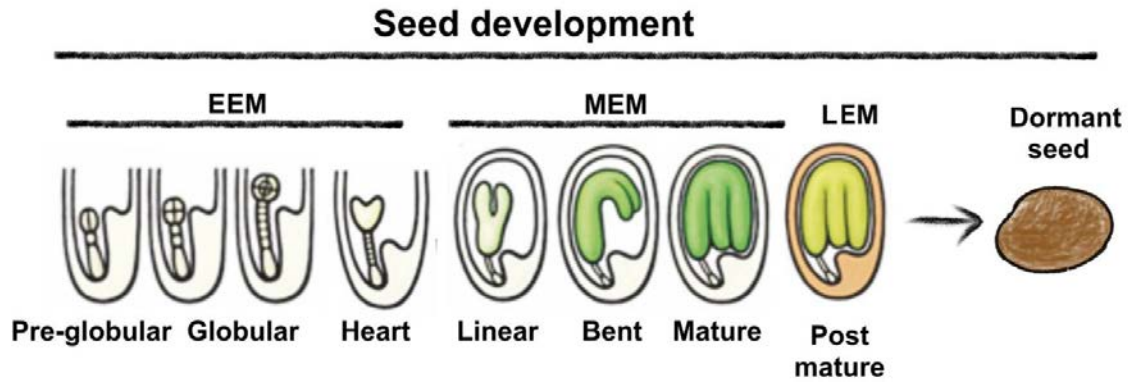


Figure 1.9 Stages of seed development. Seed development can be divided in several stages. Three of those developmental stages are depicted here. An initial stage is characterised by morphologic development, where the embryo cell division and differentiation are elevated (early embryogenesis stage, EEM). The following stages are characterised by maturation of the embryo (mid and later stages, MEM and LEM, respectively), occurring a large accumulation of reserve compounds, ABA and protective proteins. The progressive acquisition of desiccation tolerance and induction of embryo dormancy leads to the dormant seed. Adapted from Le *et al.* (2010).

In Arabidopsis, the main regulators of seed development are LEC1 (LEAFY COTYLEDON-1), LEC2 (LEAFY COTYLEDON-2), FUS3 (FUSCA3) and ABI3 (ABSCISIC ACID-INSENSITIVE 3) (Giraudat *et al.* 1992; Lotan *et al.* 1998; Stone *et al.* 2001; Chekanova *et al.* 2007). During early embryogenesis, *LEC1*, *LEC2* and *FUS3* genes are required to maintain embryonic cell fate and to specify cotyledon identity (Figure 1.10) (Santos-Mendoza *et al.* 2008). These proteins, together with ABI3 are also involved in the initiation and maintenance of maturation phase of embryogenesis. ABI3 is essential for correct completion of seed maturation and functions as a transducer of ABA induced dormancy. Indeed, ABI3 is considered as one of the major regulators of the transition between embryo maturation and early seedling development (Nambara *et al.* 1995).

The Arabidopsis mutants *lec1*, *lec2*, *fus3* and *abi3* have seeds intolerant to desiccation because of the reduced amount of compounds that are accumulated (Meinke *et al.* 1994). Indeed, during embryogenesis, respective mutant embryos display morphological features characteristic of developing seedlings. Nevertheless, there are differences among *lec1*, *lec2*, *fus3* and *abi3* phenotypes. The *abi3* mutant it is not affected at the post-embryonic development, while *lec1, lec2* and *fus3* share defects in some tissues formed at the post-embryonic phase, like trichomes and vascular tissue pattern in cotyledons (Meinke *et al.* 1994). Such phenotype suggested that *LEC1, LEC2* and *FUS3* are required for cotyledon identity and are co-regulated during embryogenesis. However, to avoid their expression through post-embryonic phase, these genes are later epigenetically repressed by PKL, a chromatin remodelling protein (Figure 1.10) (Dean Rider *et al.* 2003).

Consistent with their partial functional redundancy, the *LEC2*, *FUS3* and *ABI3* encode related transcription factors of the B3 domain family. This domain was originally identified in the maize VP1 transcription factor, a orthologue of the Arabidopsis ABI3 (Santos-Mendoza *et al.* 2008). The B3

domains of ABI3 and FUS3 are structurally similar and both transcription factors bind to RY motifs, present in many ABA-dependent inducible gene promoters. The *LEC1* gene encodes for a CCAAT-box-binding factor (CBFs) HAP3 subunit (Lotan *et al.* 1998). Both *cis*-acting motifs are present in a particular set of co-regulated genes, mostly involved in nitrogen and carbon metabolism and cell cycle.

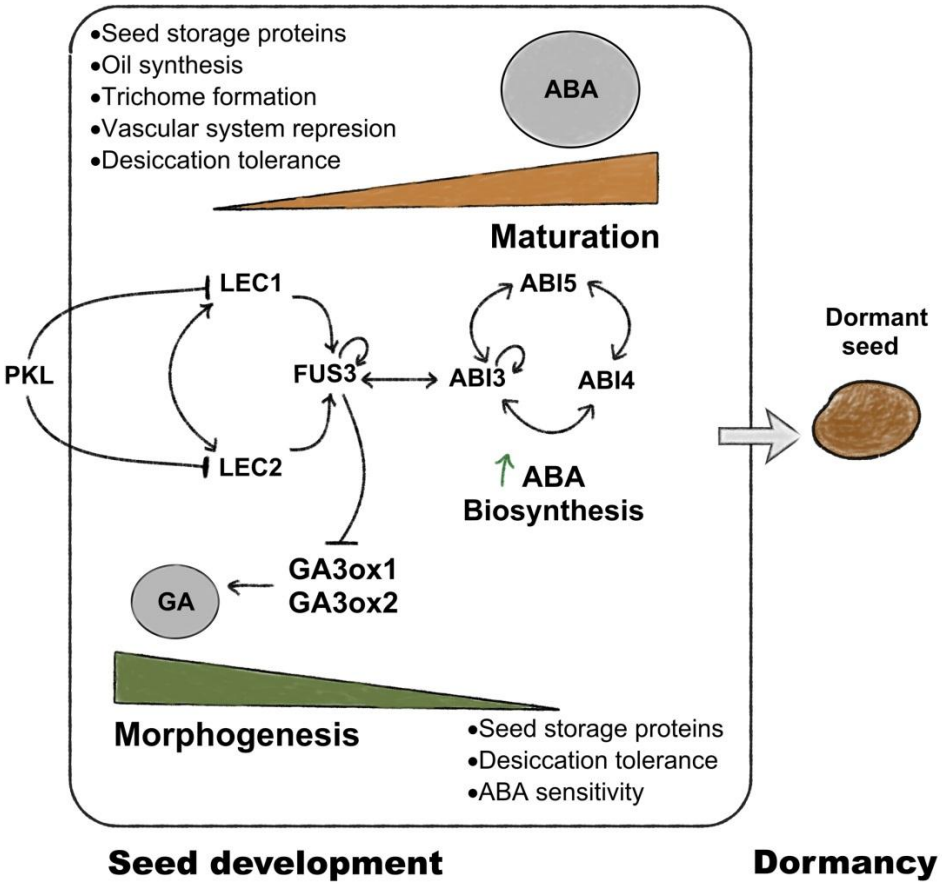


Figure 1.10 Regulation of seed development in Arabidopsis seeds and proposed interactions between some of the genes involved. Model proposed for the genetic and molecular interactions in the seed development regulatory network, in Arabidopsis. Arrows and T bars indicate positive and negative effects, respectively. Green arrow indicates up-regulation of molecular components involved in ABA biosynthesis. Brown and green triangles represent hormonal levels of ABA and GA, respectively. The different dimensions of ABA and GA hormone circles correspond to their influence during seed development. The interactions between regulators, environmental factors, hormones and different genes are described in the main text.

From morphogenesis until maturation of embryo, the levels of ABA and bioactive GAs are thought to be negatively correlated (Figure 1.10). This tight control of ABA/GA ratio is guaranteed by specific regulators involved in signalling and metabolism of these hormones (Razem *et al.* 2006). During the early stages of embryogenesis the biosynthesis of GA is important to maintain the proper embryo growth, preventing seed abortion (Singh *et al.* 2002). In Arabidopsis, the bioactive GAs are produced by gibberellins oxidases (GAox), the AtGA20ox and the AtGA3ox enzymes, which are encoded by gene families composed of five and four genes, respectively (Figure 1.10). Previous

work demonstrated that *AtGA3ox1*, *AtGA3ox2* and *AtGA3ox4* were induced in early immature seeds and their spatial and temporal expression pattern was different in embryo meristems (Mitchum *et al.* 2006). The *AtGA2ox6* was shown to be highly expressed until the end of embryo morphogenesis (Wang *et al.* 2004a; Kim *et al.* 2005; Mitchum *et al.* 2006).

The metabolic switch between GA and ABA occurs when the embryo enters the maturation stage and is transiently promoted by FUS3 (Gazzarrini *et al.* 2004). This transcription factor appears to repress the expression of *AtGA20ox1* and *AtGA3ox1* by binding to RY *cis*-elements present in their promoter sequences. This FUS3 negative regulation causes a lower synthesis of GA and an increase in ABA levels (Gazzarrini *et al.* 2004). The rising of ABA levels activates specific signalling pathways that promote the induction of important seed maturation-related genes.

During seed maturation, ABI factors (ABI3, ABI4 and ABI5) are recruited to the ABA signalling pathway. At this stage, the interplay between ABI factors is crucial to regulate the transition between the two peaks of ABA production that occur during embryo maturation. In the first peak, ABA is synthesised in both embryo and maternal tissues, whereas in the second peak, ABA only rises on embryo tissues (Finkelstein *et al.* 2002). The use of genetic approaches has allowed the identification of *abi3*, *abi4* and *abi5 mutants*, which are remarkably ABA insensitive (Holdsworth *et al.* 2008). The *abi3* mutant is strongly intolerant to desiccation, when compared to *abi4* and *abi5*. The *abi4* and *abi5* seeds display a desiccation tolerance similar to wild-type (Reeves *et al.* 2011). The *ABI4* encodes an AP2-type transcription factor that binds the coupling element1 (CE1) in promoters. The CE1 acts cooperatively with G-box-like ABA-responsive elements (ABREs), mostly present in promoters of ABA-responsive genes (Reeves *et al.* 2011). Moreover, these *cis*-elements also co-exist with RY motifs in genes whose transcripts are highly accumulated in dry seeds. *ABI5* gene encodes a b-ZIP transcription factor that is capable of binding to ABREs. Transcriptomic data suggest that ABI5, cooperatively with ABI3 and ABI4, are essential in determining the composition of mRNAs that will be stored in Arabidopsis dry seeds, suggesting a co-regulation and interaction of ABI factors (Nakabayashi *et al.* 2005) (Figure 1.10).

The metabolic regulation of ABA levels is achieved by expressing genes implicated in the biosynthesis or deactivation of ABA during seed development and germination. The main enzymes in ABA biosynthesis pathway are encoded by *NCED* genes (9-cis-epoxycarotenid dioxygenases). The Arabidopsis genome contains five possible *NCED* genes (Yamaguchi *et al.* 2007). Previous works demonstrated that NCED6 and NCED9 are the major isoforms involved in regulating seed development (Lefebvre *et al.* 2006).

1.5.2 Regulation of seed germination potential

Germination corresponds to the period that comprises the start of dry seed imbibition until the emergence of the embryo (usually through of radicle) from the enclosing tissues (Nonogaki *et al.* 2007). Important physiological, metabolic and molecular events occur during germination. These events mostly depend on environmental conditions: light, temperature and nutrient conditions. Indeed, the germination also appears to be mediated by a hormonal balance between ABA and GA (Razem *et al.* 2006). Accumulating evidences indicate that GA is the principal hormone controlling germination, through integration of light and temperature conditions (Figure 1.11).

The light-dependent pathway of germination induction is under control of phytochromes. The first evidence was provided by Borthwick *et al.* (1952) when showed that dark-imbibed lettuce seeds radiated with red (R) light germinated but not when a far-red (FR) light was imposed. In Arabidopsis, the phytochrome PHYB is stored in seeds at maturity and is responsible for typical photoreversible responses during imbibition (Shinomura *et al.* 1994; Shinomura *et al.* 1996). Accordingly, in the *phyB* mutant, the *AtGA3ox1* and *AtGA3ox2* expression is not increased by R-light. This demonstrates the role of PHYB in the regulation of GA3-oxidases gene expression (Figure 1.9) (Yamaguchi *et al.* 1998; Mitchum *et al.* 2006). However, a phytochrome-interacting protein, PIL5, has been shown to function as a negative regulator of seed germination (Oh *et al.* 2004). PIL5, a basic helix-loop-helix protein, is one of the major components linking light signals to GA metabolism and responsiveness. This light-labile protein seems to be partly related with transcriptional repression of *GA3ox* genes, in darkness. Indeed, the reduction of PIL5 proteins levels seems to be crucial in regulation of two *DELLA* genes (GA repressors), during seed germination (Oh *et al.* 2004; Oh *et al.* 2007). DELLA proteins belong to a subfamily in the GRAS family of putative transcription factors. In Arabidopsis, DELLA comprise five genes: *RGA (REPRESSOR OF ga1-3), GAI (GA INSENSITIVE), RGL1 (RGA-LIKE1), RGL2 and* RGL3 (Sun 2008). After GA sensing by the soluble receptor GID1 (GA-insensitive Dwarf1), the downstream activity induces the proteolysis of these repressors through the ubiquitin-26S proteosome pathway (Sun and Gubler 2004; Thomas and Sun 2004). *RGA* and *GAI* repress stem elongation, while *RGL1*, *RGA* and *RGL2* repress the flowering (Itoh *et al.* 2003). From all, *RGL2* has been shown to encode the major negative regulator of seed germination (Lee *et al.* 2002; Tyler *et al.* 2004; Cao *et al.* 2005). The SPY protein, has revealed as being an important regulator of GA signalling. The SPY protein has been described as a negative regulator of plant GA responsiveness, possibly involved in the alteration of activity or stability of DELLA proteins (Qin *et al.* 2011). Future

investigations are necessary to further uncover the molecular relationships between light signalling components and GA biosynthesis genes.

Temperature is other crucial environmental factor that controls seed germination. The exposition of seeds to cold temperature (stratification) promotes seed dormancy in many plants. Previous works have demonstrated that cold treatment (essentially the pre-incubation at cold temperature in dark) is a potential regulator of GA biosynthesis. In Arabidopsis seeds, during dark-imbibition at 4ºC, the level of both *GA3ox1* and *GA3ox2* mRNAs greatly increased (Yamauchi *et al.* 2004). Indeed, the increase of bioactive GAs, the cold-induction of GA biosynthesis genes and the germination ability were compromised in the *ga3ox1* mutant, demonstrating that GA3ox1 is important for temperature sensing (Yamaguchi and Nambara 2007). Inversely, the expression of *GA2ox2, a* GA deactivation gene is diminished, during dark-imbibition at 4ºC

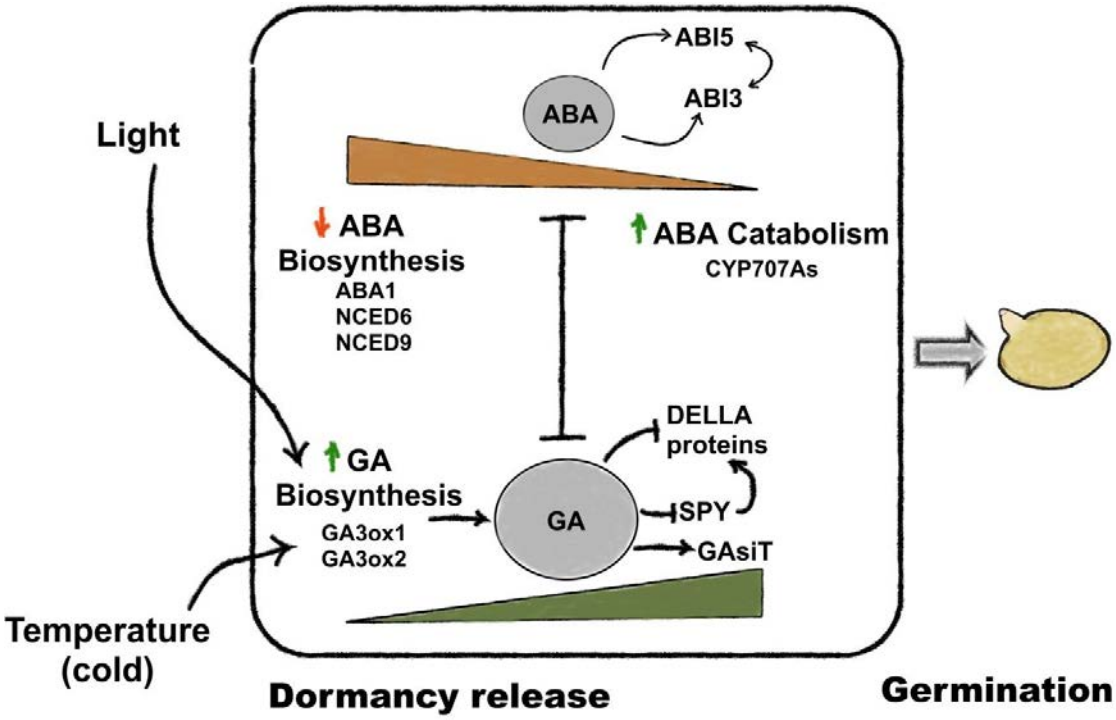


Figure 1.11 Regulation of seed germination in imbibed Arabidopsis seeds and proposed interactions between some of the genes involved. Model proposed for genetic and molecular interactions in imbibed mature seeds in Arabidopsis. Arrows and T bars indicate positive and negative effects, respectively. Green and red arrows correspond to up- and down-regulation of molecular components involved in each metabolic pathway, respectively. Brown and green triangles represent hormonal levels of ABA and GA, respectively. The different sizes of ABA and GA hormone circles relates to their respective influence during seed germination. GAsiT, GA signalling transduction. The interactions between regulators, environmental factors, hormones and different genes are described in the main text.

Besides a GA increase after seed imbibition, the levels of ABA decrease as result of the activity of deactivation enzymes CYP707As, which catalyse the ABA 8'-hydroxylation. In Arabidopsis, there are four members of *CYP707As* (Kushiro *et al.* 2004). These genes are

differentially expressed during seed germination, suggesting that each member has distinct physiological roles, being responsible by control of ABA levels during early stages of germination. During the first two phases of imbibition (rapid water uptake and plateau, respectively), a brief increase of ABA levels occurs, that requires ABI5 (Figure 1.11). ABA appears to regulate the ion-channel activities and aquaporin expression and abundance (Kucera *et al.* 2005). In the third phase of imbibition (water uptake that promotes the embryo elongation and radicle emergence) the degradation of ABI5 occurs. This event is regulated by ABI5 interaction with AFP (ABI five-binding protein), which is also induced by ABA (Lopez-Molina *et al.* 2001). The progressive decrease of ABA biosynthesis and sensing is followed by the endosperm rupture, embryo extension and seedling growth after the radicle emergence. This particular regulation of ABA levels, in parallel with GA levels rising, promotes the environmental adaptation of the embryo during the early stages of seed germination.

The fundamental knowledgement about the different developmental regulators and signalling pathways involved in seed development and germination is crucial for future improvement of seed quality in crop plants, namely under extreme temperature conditions.

1.6 Principal aims of thesis

The large quantity of transcriptomic data provided by ATH1 Gene Chip experiments, deposited in NASCArrays (NASC International Affimetrix Service), allowed the selection of several heat-responsive genes (Silva-Correia 2009). In that study, a search for heat-determinants was conducted by the use of the "heat stress time course experiment", from the "AtGenExpress Abiotic Stress Series". In this transcriptomic experiment Arabidopsis seedlings (roots and leaves) and suspension cells were heat-stressed and allowed to recover. After an extensive bioinformatic analysis, an uncharacterised gene (*HRR, At5g53680*) seemed to be more specific to HS responses and was selected for further studies.

The *HRR* gene encodes a RNA-binding protein that could be involved in transcript binding during heat stress, thus representing a putative determinant gene for thermotolerance. The principal goal of this thesis is to functionally characterise the *HRR* gene. The work will be performed in the plant model *A. thaliana*, using bioinformatic, phenotypic, molecular and cellular approaches. A bioinformatics analysis will be performed for prediction of putative HRR functions, considering its structural and phylogenetic relationships with other Arabidopsis homologues and metazoan orthologues. In addition, the bioinformatic data obtained from analysis of transcriptomic data and *cis*

promoter elements will corroborate the global prediction of *HRR* inducibility upon HS and will give new information about *HRR* expression profile in different plant tissues, plant development stages, mutants and other stressful conditions. The use of knockout (*hrr*) and HRR over-expression mutant lines will be used for studying the possible involvement of HRR in abiotic stress responses and in regulation of physiological levels of ABA and GA phytohormones. For corroborating previous bioinformatic data, the expression profile of *HRR* gene will be analysed under HS conditions, as well as during seed development and germination. In order to understand the possible involvement of HRR in regulation of several transcripts, an expression analysis of genes related to HS responses, seed development and germination will be performed in wild-type *Ler*, *hrr* and HRR over-expression mutant lines. The functional analysis of HRR will be complemented with subcellular analysis of HRR proteins, in order to understand their cellular targets and dynamics. Altogether, the results obtained from this thesis are expected to provide new insights about HRR involvement in plant thermotolerance, seed development and germination processes.

2. MATERIAL AND METHODS

2.1 *In silico* analysis of *HRR*

2.1.1 Blast searches

Blast searches were performed using the HRR protein sequence in *TBLASTN* program at NCBI (*The National Center for Biotechnology Information*, http://www.ncbi.nlm.nih.gov/) and *WU-BLAST* at TAIR (The *Arabidopsis Information Resource*, http://www.arabidopsis.org/). All sequences of Arabidopsis RRM-containing proteins were obtained from NCBI protein database.

2.1.2 Conserved domains analysis

The search for protein conserved domains on HRR sequence was performed by using the NCBI Conserved Domains tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and the Pfam database of the *Wellcome Trust Sanger Institute* (http://pfam.sanger.ac.uk/).

2.1.3 *Cis*-regulatory elements

The prediction of *cis*-regulatory elements on the promoter sequence of *HRR* was conducted on *ActisDB* platform, residing in the *Arabidopsis Gene Regulatory Information Server* (AGRIS, http://arabidopsis.med.ohio-state.edu/) and Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl).

2.1.4 Expression profiles

Expression patterns of *HRR* transcripts were predicted through BAR - *The Bio-Array Resource for Plant Biology* (http://142.150.214.117/welcome.htm) and Genevestigator (https://www.genevestigator.com/) platforms, using the BAR *Arabidopsis* and *Cell eFP* tools, or the Genevestigator *Meta-profile* analysis and *Clustering* analysis tools. ATTED II- *Arabidopsis thaliana trans-factor and cis-element prediction database* (http://atted.jp/), Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) and GeneMania (http://www.genemania.org/) platforms were used to predict co-expression and co-localisation networks.

2.2 Phenotypic characterisation of *hrr l*oss-in-function and HRR over-expression mutant lines

2.2.1 Plant material and growth conditions

The *HRR* mutant line (*hrr* mutant), a GT_5_47364 transposon line in *Landsberg erecta* (*Ler*) background of *A. thaliana*, was used to evaluate the effect of *HRR* (*At5g53680*) loss-in-function. This line and wild-type *Ler* were obtained from John Innes Centre (JIC, UK) collection and ordered through NASC center (http://arabidopsis.info/). Plant growth was promoted under a long photoperiod (16 h light/ 8 h dark), at 23ºC with 80 µE $m^{-2}.s^{-1}$ of light intensity (Annex II, sections 1 and 2).

2.2.2 Plasmid construct and plant transformation

For producing over-expression lines, the *p35S::HRR-GFP6* construct was obtained through the Gateway system (Invitrogen). RNA from 16-days-old *Ler* seedlings, subjected to HS for 60 min at 38ºC, was used for the cDNA synthesis and amplification of the *HRR* coding sequence (510 bp). PCR conditions and specific primers are presented in Annexes III and IV. The amplified sequence was cloned into the *Gateway*® vector pDONR™201 (Invitrogen) by performing the BP recombination reaction (Annex II, section 12). The resulting pENTR-HRR vector was used to perform the LR recombination reaction with the pDEST vector pMDC83 (Curtis and Grossniklaus 2003), which contained the translational fusion GFP6 (C-terminal GFP6). The *Agrobacterium tumefaciens* (EHA105 strain) harbouring the resulting *p35S::HRR-GFP6* construct was used to transform wild-type (*Ler*) and *hrr* mutant Arabidopsis plants by the floral dip method (Clough and Bent 1998). Single genetic transformants were detected in the T2 generation by growing on MS medium supplemented with hygromycin and followed a 3:1 segregation. From the homozygous T3 transgenic lines, five transformed lines were selected: L2 and L6 (in *Ler* genetic background), and JP5, JP6 and JP9 (in *hrr* genetic background). Synchronised plants of *hrr* and HRR over-expression mutant lines (T3), as well as wild-type *Ler*, were screened by diagnostic PCR using the conditions and specific primers described in Annexes II (section 8), III and IV.

2.2.3 Selection of HRR homozygous recessive insertion and over-expression lines

About 100 plants from the T3 generation of ordered *hrr* seed stocks were grown for diagnostic PCR and seed harvesting. Genomic DNA from Arabidopsis leaves was extracted (Annex II, section 3) and used for selecting the homozygous *hrr* mutant lines by diagnostic PCR analysis (Annex II, section 8). For this amplification a multiplex primer system was used: one specific primer for the transposon insertion (*prb.ZF*_rv primer) and two primers conceived to *HRR* gene (*RB* and *LB* primers). These primers were designed using the *Oligo6* software (*Primer Analysis* Software, version 6.68). Primer sequences and PCR conditions are present in Annexes III and IV, respectively. After gel electrophoresis (Annex II, section 7), the *hrr* mutant seeds from those plants displaying the proper estimated fragment size were harvested. The diagnostic PCR analysis was repeated for these *hrr* mutants in the following three generations to guarantee that transposon insertion remained stable. From the selected homozygous T3 HRR over-expression lines, a diagnostic PCR was performed to evaluate the presence of *p35S::HRR-GFP6* transgene in the genome of these plants. The genomic DNA was isolated and amplified with diagnostic primers for pMDC 35S and pMDC gfp left borders as referred above.

2.2.4 Expression analysis of HRR homozygous recessive insertion and over-expression lines

To analyse the *HRR* expression levels in five-weeks-old plants of wild-type (*Ler*) and *hrr* mutant lines, grown under standard conditions, total RNA was isolated from different Arabidopsis organs/tissues with Trizol® reagent (Annex II, section 4). For the cDNA synthesis (SuperScript First-Strand Synthesis System, Invitrogen), it was followed the provider instructions (Annex II, section 9). The resulting cDNA pools were then used for the gene expression analysis by semi-quantitative RT-PCR amplification, which was performed as described in standard protocol for PCR reaction (Annex II, section 8). The same procedure was followed for confirming the over-expression of *HRR* transcripts in HRR over-expression seedlings (16-days-old), grown under standard conditions. The gene-specific primer pairs used for this analysis, HRRcDNA_fw/HRRcDNA_rv for wild-type *Ler* and *hrr* mutant sample and HRR_RT_fw/HRR_RT_rv for HRR over-expression lines, and corresponding PCR conditions are presented in Annexes III and IV, respectively. The constitutive gene *Actin2* (*ACT2*)

expression levels were simultaneously analysed as internal control for RNA amount normalisation of each RNA sample (gene-specific primers and PCR conditions are presented in Annexes III and IV).

2.2.5 Thermotolerance germination assays

All seed germination assays were performed with synchronised seeds (seed pools from lines simultaneously grown in the same conditions). Seeds were stratified (4ºC, 2 days) in the dark, and subsequently surface sterilised (Annex II, section 1). For HS treatments, sterilised seeds were heat-stressed by immersion of respective microtubes into a water bath under a constant temperature of 50°C, for different periods (15-300 min), or at different temperatures (38-56°C) for 60 minutes. Immediately after HS, seeds were resuspended in sterile 0.25% (w/v) agarose solution and sown onto MS-agar medium. The plates were incubated under 16h light/8h dark photoperiod (80 $\mu E.m^{-2}.s^{-1}$ light intensity) at 23ºC. The emergence of radicle was followed every day, from second to tenth day after stress imposition. Germination rate (as percentage, %) was normalised with corresponding germinated seeds in control conditions (23ºC). Mean and SEM were determined based on results from four replicates for each seed line, all containing 30 seeds. Results were submitted to statistical analysis using *t*-test, one-way or two-way analysis of variances (ANOVA) tests (*GraphPad Prism* v.5 program), considering statistically significant differences those that exhibit p-values of <0.001, <0.01 or <0.05. All experiences were repeated with similar results.

2.2.6 Salt, osmotic and oxidative stress assays

Germination of heat stressed seeds in salt, osmotic or oxidative stress conditions was performed as previously referred, but with some modifications. Stratified *hrr* mutant and wild-type *Ler* seeds were heat-stressed (47ºC), during for 60 min. Seeds were sown and allowed to germinate onto MS medium supplemented with different concentrations of sodium chloride (NaCl; 0, 75, 100, 150, 200 mM), mannitol [1.5, 3, 5, 7% (w/v)] or paraquat (0.001, 0.01, 0.1, 1 µM). The appearance of green and fully expanded cotyledons (viable seedlings) was scored at 10th day of assay. The same assay was simultaneously performed with stratified seeds without HS treatment. Survival seedlings percentage (survival rate) was normalised with corresponding viable seedlings in control conditions (non-HS seeds sown onto MS medium without stressors). Mean and SEM were determined based on results from three replicates for each seed line, all containing 40 seeds. Statistical analysis was performed as described for thermotolerance germination assays.

2.2.7 Hormonal sensitivity assays with ABA and GA_3

Germination of seeds in the presence of exogenous ABA and an active form GA_3 was performed as previously described, with some modifications. Stratified wild-type *Ler, hrr* and HRR over-expression mutant seeds were sown onto MS medium supplemented with different concentrations of ABA (0.5, 1, 1.5 and 2 µM, Duchefa). For germination assays in the presence of exogenous GA_3, stratified *hrr* mutant and wild-type *Ler* seeds were directly sown onto MS-agar medium containing variable GA_3 concentrations (25, 50, 75 and 100 µM, Duchefa). Whenever a HS treatment was applied, stratified seeds were previously heat-stressed at 50ºC, during 60 min. The emergence of radicle was scored every day, from the second to the tenth day after sowing. Germination rate (%) was normalised with respective viable seeds in control samples. Mean and SEM were based on results from three replicates for each seed line, all containing 40 seeds. Statistical analysis was performed as described for thermotolerance germination assays.

2.3 *HRR* gene expression and their putative roles in regulation of HS- and plant developmental-related transcriptomes

2.3.1 Biological samples and treatment conditions

2.3.1.1 Heat stress treatment on Arabidopsis seedlings

To analyse the *HRR* expression during HS treatment, wild-type *Ler* and *hrr* mutant seedlings (five-six seedlings, 16-days-old) were heat-stressed at 38ºC, for 15 to 180 min, followed by a recovery period (15, 30, 60, 120 and 180 min) at control conditions (23ºC). After HS treatment and recovery period, each sample was immediately frozen in liquid N_2. For *in vivo* analysis of mRNA decay of *HRR* alternative transcripts, wild-type *Ler* seedlings (16-days-old) were either untreated (23ºC) or heat-stressed at 38ºC for 60 min. All procedures corresponding to RNA and cDNA manipulation for these samples are described in Annex II (sections 4 and 9, respectively).

In case of histochemical analysis of *HRR* promoter activity, wild-type *Ler* and transgenic *pHRR::gusA* seedlings (seven-days-old) were HS-treated (38ºC), during three hours. Transgenic *p35S::gusA* were directly subjected to GUS assay (without HS treatment, 23ºC).

2.3.1.2 Seed and siliques material treatment

To analyse the *HRR* expression during seed maturation and germination, siliques, stratified seeds, and germinated seeds from wild-type *Ler* and *hrr* mutant lines were used. The whole siliques were harvested during the seed maturation stage, according with the embryo and seed development stages considered in *Arabidopsis eFP Browser*. Desiccated seeds (from 12-weeks-old plants) were stratified (two days, at 4ºC), being further heat-stressed in water bath (50ºC, 60 min) or maintained at standard conditions (23ºC). This last procedure was also made for HRR over-expression lines. For getting germinated seeds, stratified and sterilised seeds were sown onto MS medium and were harvested after the first and second days of sowing. All procedures corresponding to RNA and cDNA manipulation for these samples are described in Annex II (sections 5 and 9, respectively).

2.3.2 *In vivo* analysis of nonsense-mediated mRNA decay of *HRR* transcripts

To analyse if the *HRR* transcripts are removed *in vivo* by NMD mechanisms, the methodology reported by Hori and Watanabe (2008) was followed with some modifications. Immediately after the HS treatment (section 2.3.1.1), four to six seedlings from each treatment were rinsed in a 2 ml microtube containing MS medium, supplemented with the appropriate inhibitor: 100 µg.ml^{-1} Actinomycin D (ActD, Biochemia) or 20 µM cycloheximide (CHX, Merck). Controls were prepared using the same procedure but with no inhibitor supplementation. The seedlings were slightly wounded and held down with the micropestle. The samples were immediately put under vacuum for 7 min. After infiltration, the medium was removed and the samples immediately frozen in liquid N_2. All samples were ground in liquid N_2 and used for RNA purification (Annex II, section 4). Total RNA (1 µg) was used for first strand cDNA synthsis (Annex II, section 9).

2.3.3 Histochemical analysis of *HRR*

2.3.3.1 HRR promoter cloning into pCAMBIA and plant transformation

The cloning of *HRR* promoter sequence in fusion with *gusA* coding sequence (present in the pCAMBIA1303 vector, Annex V) allows the histochemical analysis of *HRR* promoter activity, in different Arabidopsis organs and under HS conditions. The *HRR* promoter sequence was amplified from *Ler* genomic DNA (Annex II, sections 3 and 8), using specific primers that added the restriction

sequences *Hind*III and *Bgl*II in the PCR products (Annex III). Purified PCR products and pCAMBIA1303 vector were restricted with the referred enzymes, during 4 hours, at 38ºC (Annex II, section 13). After restriction and purification, both fragments were used for T4 DNA ligation reaction (Annex II,14) and 5 µl of ligation reaction was used to transform XL1-Blue *E.coli* competent cells (Annex II, section 16). The transformants were grown in LB-agar medium supplemented with 50 µg.ml^{-1} kanamycin. By using the specific primers used for cloning into pCAMBIA1303, a colony PCR was done for further selected transformants, where the insert is introduced upstream to *gusA* sequence (Annex II, section 8). The PCR products were analysed by agarose gel electrophoresis and positive transformants were selected according to the expected fragment size (Annex II, section 7). After sequencing confirmation, the recombinant plasmid was used to transform *A. tumefaciens* EHA105 strain (Annex II, section 19). After selection in LB-agar medium supplemented with 50 µg.ml^{-1} rifampicin and 50 µg.ml^{-1} kanamycin, ten transformants were confirmed by colony PCR using the last referred specific primers. The selected recombinant plasmid were used for transformation of wild-type *Ler* plants by the floral dip method (Annex II, section 20).

2.3.3.2 Histochemical localisation of GUS *fusions and observation*

For the histochemical analysis of *HRR* expression in Arabidopsis tissues, transgenic and *p35S::gusA* (positive control) seedlings were used. After HS treatment (*pHRR::gusA* and wild-type *Ler*) or not (*p35S::gusA*), seedlings were immediately fixed in 90% (v/v) ice-cold acetone, for 5 min, on ice. After fixation, supernatant was replaced by 2 ml of X-Gluc staining solution (0.1 mM sodium phosphate pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.3% (w/v) Triton X-100, 10 mM EDTA and 1mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) [adapted from A.Jefferson *et al.* (1987)]. The samples were vaccum infiltrated at 600 mmHg for 10 min, at room temperature, four times. The samples were then incubated overnight either at 37ºC or 23ºC, depending on the GUS signal to be obtained. After these incubation periods, the samples were subsequently washed with 90% (v/v) ethanol, in order to remove chlorophyll from plant tissues. The seedlings were mounted on microscope slides and visualised in a *Leica DM 5000 B* microscope, under bright field.

2.4 Subcellular dynamics of HRR proteins: perspectives on functional roles

2.4.1 pGEM®-T Easy Cloning of HRR.2 isoform

The PCR products corresponding to alternative-spliced *HRR.2* trasncript were cloned into pGEM®-T Easy vector (Promega). The *HRR.2* PCR products were first purified from agarose gel (Annex II, section 11) and subjected to A-tailing reaction. As the *Pfu* polymerase only produces blunt-end fragments, the *HRR.2* products (7 µl) was mixed with 1 µl 10x reaction buffer (with $MgCl_2$) (Promega) and dATP (Promega) to a final concentration of 0.2 mM. After thoroughly mixing, 5U of Taq polymerase and double-distilled water were added for a final reaction volume of 10 µl. The reaction was incubated at 70ºC for 30 min and then kept onto ice until the cloning. The ligation was performed as described in Annex II (section 14). The reaction and corresponding control were incubated at 4ºC, overnight, after which 2 µl were directly used to transform XL1-Blue *E. coli* competent cells (Annex II, section 15). Each transformation culture (50 µl) was plated onto duplicate LB medium plates, containing 100 $\mu g.ml^{-1}$ ampicilin, 100 mM IPTG and 50 $mg.ml^{-1}$ X-Gal. The plates were incubated at 37ºC, during overnight. Then, the plates were screened for white colonies (15), which usually contain a successful cloned insert, and selected and subsequently screened by colony PCR (Annex II, section 8), using the *HRR.2* insert-specific primers (Annex III). After confirmation, this construct was used as template for all *HRR.2*-containing cloning procedures.

2.4.2 Cloning strategy

The cloning strategy used to obtain the of *HRR.1* and *HRR.2* fusion constructs was based in the Gateway® Technology (Invitrogen) (Annex VI). To obtain the ectopic *HRR.1* and *HRR.2* constructs in fusion with *GFP6* sequence, it was performed the following recombination reactions. The *HRR.1* sequence (510 bp) was insert in pMDC43 and pMDC83 vectors (Annex V), producing the N- and C-termini fusions, respectively. Two *HRR.2* sequences (583 and 257 bp) were respectively inserted in pMDC43 and pMDC83 vectors, then originating the N- and C-termini fusions (Curtis and Grossniklaus 2003). The predicted 650 bp promoter region (AGRIS source) was cloned in pMDC43 (using *Hind*III and *Kpn*I) and into pMDC83 (using *Hind*III and *Spe*I). These restriction enzymes were selected to replace the double CaMV 35S promoter (Annex II, section 13). The resulting *pHRR*

destination vectors were used to clone the *HRR.1* and *HRR.2* cDNA sequences by LR recombination process. The donor and destination vectors were ordered from ABRC (http://abrc.osu.edu/).

2.4.2.1 Ectopic expression of HRR.1 and HRR.2 in fusion with GFP6

The *HRR.1* and *HRR.2* sequences were amplified and flanked by *att*B recombination sites in two-round PCR amplifications using as template cDNA from HS-treated *Ler* seedlings (16-days-old, under 38ºC, during 60 min). The primers used to amplify these sequences were designed in *Oligo6* software (*Primer Analysis* Software, version 6.68) and are presented in Annex III. The *att*B PCR products from *HRR.1* and *HRR.2* were initially amplified with sequence-specific primers, which contained 12 nts of *att*B1 and *att*B2 recombination sites coupled at each 5' end (Annex II, section 8). The second PCR was performed to complete the *att*B1 and *att*B2 full sequences, employing then the adapter primers (Annex III). The PCR conditions are presented in Annex IV. The *att*B PCR products were subsequently purified from agarose gel (Annex II, section 11). The BP recombination reaction was accomplished using 100 fmol of each *att*B-PCR product and pDONR™201 (Annex II, section 12). After incubation, 5 µl of each resulting pENTR vectors was used to transform XL1-Blue *E.coli* competent cells (Annex II, section 16) and the transformants were grown in selective LB-agar medium, supplemented with 50 µg.ml^{-1} kanamycin. From resulting transformants, 24 isolated colonies were selected to perform a colony PCR to evaluate the transformation efficiency, using the primers pDON201Seq (Annex II, section 8; Annex III). Positive transformants were selected according to the expected fragment size. After isolation of plasmid DNA (Annex II, section 17) and sequencing confirmation, cloned sequences were used to perform the LR recombination reactions (Annex II, section 12). XL1-Blue *E.coli* competent cells were transformed with resulting pEXP vectors (*p35S::GFP6-HRR.1, p35S::GFP6-HRR.2, p35S::HRR.1-GFP6his, p35S::HRR.2-GFP6his*). Positive transformants were selected and identified by colony PCR, using the specific primers for confirming the Gateway LR cloning reactions (Annex II, section 8; Annex III).

After confirmation by sequencing, the new expression constructs were used to transform *Agrobacterium tumefaciens* EHA105 strain for proceeding with *A. thaliana* transformation (Annex II, section 19). After selection in LB-agar medium supplemented with 50 µg.ml^{-1} rifampicin and 50 µg.ml^{-1} kanamycin, 10 transformants were verified by colony PCR using the last referred specific primers.

2.4.2.2 Native expression of HRR.1 and HRR.2 in fusion with GFP

The destination vectors harbouring *HRR* promoter sequence in fusion with *GFP6* reporter gene were obtained to analyse the native expression of HRR.1 and HRR.2 proteins. *HRR* promoter sequence was amplified using *Ler* genomic DNA using primers containing the appropriated restriction sequences for cloning. For obtaining GFP N-termini fusion (using pMDC43 vector), primers contained *Hind*III and *Kpn*I restriction sites and for C-termini fusion (using pMDC83 vector) primers contained *Hind*III/*Spe*I restriction sites. The primers used to amplify that sequence were too designed in *Oligo6* software (*Primer Analysis* Software, version 6.68) and presented in Annex III. The resulting PCR product and pMDC43/pMDC83 vectors (Annex V) were double digested with corresponding enzymes, during four to five hours, at 37ºC (Annex II, section 13). The vectors were subsequently desphosphorylated on their 5' ends by Shrimp Alkaline Phosphatase (SAP, 1U), supplementing the restriction reaction. The reactions were deactivated by adding loading buffer and analysed by agarose gel electrophoresis (Annex II, section 7). The digested fragments were purified and used to perform a T4 DNA ligation reaction (Annex II, section 14). After incubation, the 3 µl of reaction was used to transform *ccd*B survival *E. coli* competent cells, whose transformants were selected onto LB-agar medium containing 50 µg.ml^{-1} kanamycin and 34 µg.ml^{-1} chloramphenicol (Annex II, section 16). The positive clones a were confirmed by colony PCR (Annex II, section 8) using the *HRR* promoter specific primers (Annex III). After plasmid DNA purification LR recombination reactions were performed with pENTR vectors obtained previously. The subsequent steps of transformation and selection of *E.coli* and Agrobacterium clones were done as indicated in Annex II (sections 16,17 and 19) The selected Agrobacterium clones were used to transform BY2 cells (section 2.4.4).

2.4.3 Agroinfiltration of *Nicotiana benthamiana*

Agrobacterium tumefaciens EHA105 strain cells were transformed with the expression vectors and used to perform the transient over-expression of HRR.1 and HRR.2 GFP-fusion proteins in *Nicotiana benthamiana* leaves (Sparkes *et al.* 2006).

An aliquot (20 µl) of the appropriated transformed Agrobacterium glycerol stock was placed in 7 ml LB medium, supplemented with 50 µg.ml^{-1} rifampicin and 50 µg.ml^{-1} kanamycin. After overnight growing at 28ºC, with constant shaking (200 rpm) the *A. tumefaciens* cells were resuspended in 5 ml infiltration buffer (10 mM $MgSO_4$, 10 mM MES pH 5.7, 400 µM acetoseryngone). The mixture was then infiltrated in the abaxial surface of the *N. benthamiana* leaves (one-month-old) and plants were

incubated in darkness for two days, at room temperature. The plant tissue was then mounted on microscope slides and observed at fluorescence microscope (*Leica DM 5000B*).

2.4.3 Transformation of *Nicotiana tabacum* Bright Yellow-2 (BY2) cells

Nicotiana tabacum Bright Yellow-2 (BY2) cells (kindly provided by Rita Abranches, ITQB, Lisboa) were sub-cultured at least twice before they were used as source for transformation. Three or four days before transformation, a new subculture was established by diluting a aliquot of 1.5 ml BY2 cell suspension culture in 20 ml MS medium [4.3 $g.L^{-1}$ Murashige and Skoog basal medium, 30 $g.L^{-1}$ sucrose, 0.1 $g.L^{-1}$ myo-inositol, 1 $mg.L^{-1}$ thiamine-hydroxychloride (HCl), 0.2 $mg.L^{-1}$ 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.2 $g.L^{-1}$ KH_2PO_4, pH 5.8]. The day before doing the transformation, 20 µl of glycerol stock of Agrobacterium (harbouring the appropriated construct) was inoculated into 5 ml LB medium supplemented with 50 $\mu g.ml^{-1}$ rifampicin and 50 $\mu g.ml^{-1}$ kanamycin. After overnight growing at 28ºC, with shaking at 200 rpm, the Agrobacterium cultures were harvested and centrifuged at 5000 *g* for 10 min, at room temperature. Cells were resuspended in 3 ml MES buffer (50 mM MES pH 5.7, 10 mM $MgSO_4$). Aliquots of 15 ml BY2 cells were first incubated with 500 µM acetoseryngone for 15 min, with gently mixing by swirling, during 15 min. BY2 cells were then placed into Petri dishes and 100 µl of Agrobacterium cells suspension were added. For each construct, at least two transformations were performed. The Petri dishes were gently mixed, wrapped with Parafilm and incubated on dark, at 25ºC, for two days.

The transformed BY2 cells were transferred to a 50 ml centrifugue tube with a wide-bore 10 ml pipette. The plate was additionally rinsed with 5-7 ml of MS medium, which was then added to the centrifuge tube. The BY2 cells were centrifuged at 400 rpm for 3 min and the supernatant discarded and the BY2 cells were resuspended with MS medium to a final volume of 15 ml gently mixed and centrifuged again in same conditions. This washing was repeated three times. At the end, the washing was repeated using MS medium supplemented with 500 $\mu g.ml^{-1}$ ticarcilin and cells were finally resuspended in 10 ml MS medium, supplemented with 250 $\mu g.ml^{-1}$ ticarcilin and gently mixed by inversion. After cells setting, 1 ml of washed BY2 cells were plated in selective MS-agar medium containing 250 $\mu g.ml^{-1}$ ticarcilin and 50 $\mu g.ml^{-1}$ kanamycin. As control, the same washed cells were plated in non-selective MS medium, which only contained 250 $\mu g.ml^{-1}$ ticarcilin. The cells were spread over agar surface by rocking and swirling the plate. The plates were kept open in the flux chamber for about 10 min until the liquid was absorved by the medium. After sealing with parafilm, the cells were incubated at 25ºC, in the dark for 10-14 days. At the end, visible microcalli were

transferred to a fresh MS plate, supplemented with 250 µg.ml^{-1} ticarcilin and 50 µg.ml^{-1} hygromycin. The plates were sealed and incubated in the dark, at 25ºC for eight days, to let the microcalli grow. Then they were cultured into liquid MS medium and GFP signal was detected, using in last the fluorescence microscope (*Leica DM 5000B*).

2.4.4.1 HS and chemical treatments of BY2 transformed cells

The HS and chemical treatments were performed with the transformant BY2 cells expressing the constructs *pHRR::GFP6-HRR.1* and *pHRR::GFP6-HRR.2*. For HS treatment, samples of transformant BY2 microcalli rinsed into MS medium-containing microtube, which were then incubated in a water bath at 38ºC for 15, 30 or 60 min. For performing the chemical treatments, before the HS treatment, transformant BY2 microcalli of each construct were rinsed in 100 µl of MS medium, being the cell suspensions supplemented and incubated with 100 µg.ml^{-1} cycloheximide (CHX) or 10 µg.ml^{-1} puromycin (PUR) for one hour. The cells were then subjected to HS treatment (38ºC, 60 min). As negative control, the chemical-treated cells were kept at room temperature. The positive control corresponded to transformed BY2 cells under HS treatment (60 min at 38ºC). Immediately after HS treatment, a sample was mounted on microscope slides and observed at fluorescence microscope (*Leica DM 5000 B*). The control samples, corresponding to non-transformant cells at room temperature, cells stained with DAPI and tobacco BY2 cells expressing *p35S::GFP* transgene, were only managed in MS medium.

3. RESULTS AND DISCUSSION

3.1 *In silico* analysis of HRR

In a previous work, *HRR* (*At5g53680*) was selected as being a potential genetic determinant in responses to HS imposition (Silva-Correia 2009). The *HRR* expression response profile was determined using the accessed microarray data of *Heat Stress Experiences* (AtGenExpress Abiotic Stress series) (Kilian *et al.* 2007). *HRR* seems to be strongly induced just after a heat stress imposition, in shoots and roots, as well as in cell suspensions. The maximal expression levels in suspension cells were detected after one hour of heat stress (HS, 38ºC) imposition and gradually decreased to basal values during recovery at 25ºC. In roots, *HRR* displayed an increasing expression level after one hour of HS treatment, reaching the highest levels of expression after three hours of HS (Figure 3.1). In shoots, the *HRR* expression is lower than in roots under the same conditions. During the recovery period at 25ºC, *HRR* expression decreased to basal values in all tissues.

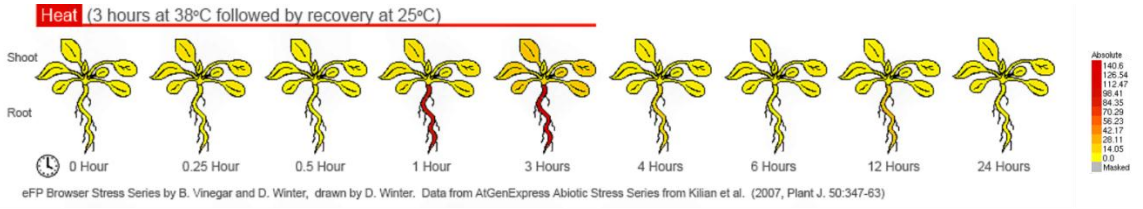


Figure 3.1 *HRR* expression profile predicted by the Arabidopsis eFP Browser under heat stress conditions. *HRR* expression levels were evaluated in heat-stressed (38ºC) *Arabidopsis thaliana* 16-d-old seedlings (roots and shoots), using data from AtGenExpress Abiotic Stress Series (NASCarrays). *HRR* displays the highest levels of expression after three hours of HS treatment. Expression levels are presented in absolute values and are identified as depicted in the color scale.

The *HRR* expression profile was additionally performed using diverse of bioinformatic tools.

3.1.1 HRR structural and phylogenetic analysis

Currently, with the raising of Arabidopsis structural and functional data, many bioinformatic tools have emerged. The major Arabidopsis bioinformatic tool, the TAIR browser (*The Arabidopsis Information Resource,* http://www.arabidopsis.org/) displays and maintains a database for the model plant Arabidopsis, besides providing useful linkouts to other Arabidopsis web resources. The data obtained from this resource indicated that *HRR* encodes a RNA-binding protein which contains a RNA Recognition Motif (RRM-containing protein) and presents an uncharacterised biological function. The *Sequence Viewer* tool in TAIR revealed that *HRR* is located in chromosome 5, in the forward strand and is composed by three exons and two introns (Figure 3.2). The coding region of

HRR is located between 21798383 and 21799109 nt and possesses a predicted promoter sequence from 21797731 to 21798381 nt. *HRR* comprises 729 bp (including introns of 73 and 145 bp), 510 bp of which codifies for the protein. The protein data, provided from different databases (Pfam, SMART, EMBL-EBI, Expasy and Panther databases), indicated that HRR harbours a RNA recognition motif ranging from 14 to 141 amino acid. This domain is found in many eukaryotic organisms, ranging from yeasts and fungi to human, and is included in many RNA-containing proteins that are implicated in RNA metabolism roles, such as splicing factors and regulators, heterogenous and small nuclear ribonucleoproteins (hnRNPs and snRNPs) and other proteins that regulate RNA stabilisation and translation (Sachs *et al.* 1987; Chambers *et al*. 1988; Query *et al.* 1989; Lorkovic and Barta 2002).

Figure 3.2 Full genomic sequence of *HRR* obtained from TAIR10 (*Sequence Viewer*). This gene is located in chromosome 5. The orange uppercase letters represent the exonic sequences. The purple lowercase letters represent the intronic sequences. Blue shaded uppercase letters represent the translational start/stop codons. Figure obtained from *Sequence Viewer* tool (TAIR).

To detail the analysis of HRR putative conserved domains, a BLAST search was performed in NCBI (*National Center for Biotechnology Information*) database, through the "*Conserved Domains*" tool (Figure 3.3). This tool allowed the alignment of HRR protein sequence against all resident sequences placed in protein databases, using the basic BLAST algorithm. Besides the above described RRM domain, HRR also holds a multi-domain PABP-1234 (polyadenylate binding protein, human type 1, 2, 3, 4). The RRM domain occurs between the K14 and R81 and the PABP-1234 domain from D9 to E148 (Figure 3.3). Human proteins described as holding a PABP-1234 domain comprises four tandem RRM domains at the N-terminus, followed by a PABP-specific domain at C-terminus. Such

proteins have been described as being involved in the recognition and transport of mature mRNAs from the nucleus to the cytoplasm (Yang *et al.* 1995 ; Afonina *et al.* 1998; Féral *et al.* 2001).

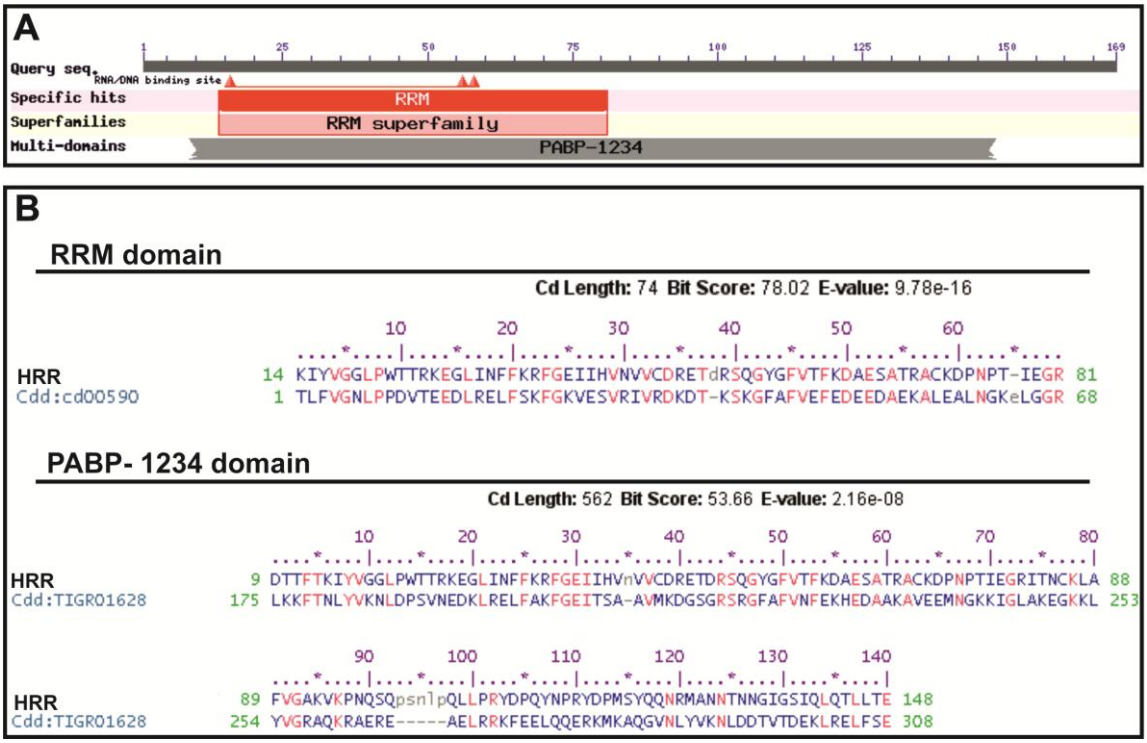


Figure 3.3 HRR protein conserved domains. (A) Protein sequence blast search identified two sequence domains: RRM domain and PABP-1234 multi-domain. (B) Both HRR protein conserved domains where aligned with the best hits for representative domain sequences. Blast search was obtained from *Conserved Domains* tool, supported by NCBI website. Identical amino acids are represented in red. The numbers refers to amino acid positions in the protein sequences.

According to the data provided by Pfam database, only the RRM domain was found to display significant match. This database also assembled this RRM-containing protein in a group of 11 related families, able to align between themselves by sequence similarity or structure. The best hits correspond to Smg4, UPF3, and RRM_3-containing protein families. The Smg4 and UPF3 proteins are involved in NMD, while the RRM_3-containing proteins, such human LA protein, function as a RNA chaperone during RNA polymerase III transcription and can also stimulate translation initiation (Aronoff *et al.* 2001; Jacks *et al.* 2003).

Collectively, these results suggest a RNA binding ability of HRR, which could even involve the binding to the poly(A) tails present in 3' ends of mature mRNAs (through its PABP-1234 domain). Putative roles on transcript stabilisation, translation initiation or, eventually, in mRNA decay processes could be predicted.

As the Arabidopsis genome codes for 196 different RRM-containing proteins (Lorkovic and Barta 2002), other Arabidopsis proteins could share similarities with HRR conserved domains. Indeed, TAIR

data revealed that the best match with HRR corresponds to another RNA-binding protein containing a RRM domain that is coded by the gene *At5g11412.1*. The WU-BLAST search (supported by TAIR) was performed using the HRR protein sequence against TAIR10 proteins database, allowing the selection of the best homologous protein sequences for HRR (Figure 3.4). The highest scores comprised two proteins, with identity percentages between 60% (for *At5g11412.1* protein) and 46% (for *At5g53720.1* protein).

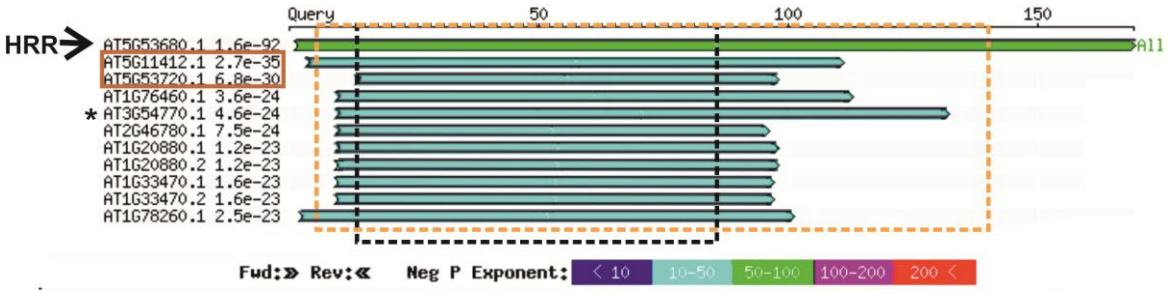


Figure 3.4 Representation of the blast search of HRR protein sequence against the Arabidopsis proteome. The 10 most homologous sequences to HRR are presented, being the closest ones enclosed in a brown box. The RRM and PABP-1234 domains are highlighted by black and orange line-dashed box, respectively. The HRR homologous protein depicted with an asterisk shares homology with PABP-1234 domain in HRR. Adapted image obtained from TAIR10 Proteins dataset of WU-Blast program in TAIR website.

As predicted for HRR, all these homologous proteins contain RNA-binding motifs and are described as being functionally uncharacterised (TAIR source). It is noteworthy to point out that the protein coded by *At3g54770* gene shares homology with HRR in almost all protein sequence queried, spanning until the PABP-1234 domain hedge. Recently, this protein was described as been involved in hormone-dependent regulation of gene expression during the transition stages of floral development (Chen *et al.* 2009). In addition, the corresponding gene has been predicted to share co-expression with other Arabidopsis PABP proteins (PABP 7, 6 and 4), according to the data displayed by GeneMANIA (data not shown).

The highest similar RRM-containing proteins with a function already attributed correspond to UBP1-associated proteins 1a (UBA1A) and UBP1-associated proteins 2a (UBA2A) proteins, displaying similarities of 41% and 33%, respectively (Figure 3.4, data not shown). These proteins, along with U-rich binding protein 1 (UBP1), have been described as being involved in nuclear mRNA stabilisation in nucleus, being components of a complex that recognises U-rich sequences in plant 3'UTRs (Lambermon *et al.* 2000; Lambermon *et al.* 2002). The predicted function of UBP and UBA proteins was based in best score alignments with metazoan hnRNPs, which have been described to play many roles in different stages of mRNA maturation: initial binding to nascent primary mRNA, regulatory tasks during splicing, mature mRNA transport, translation, and stability (Krecic and Swanson 1999).

Although the UBP and UBA proteins harbour more than one RRM domain (Peal *et al.* 2011), which most probably leads to differences in RNA binding proprieties from HRR, the structural similarity among them could suggest some functional relationships as hnRNP-like proteins.

As the functional role of RNA-binding proteins has been more studied in metazoan organisms, a new BLAST search was performed using the *UniGene* database resource, deposited in NCBI. This database provides sets of transcripts that appear to come from the same transcription locus, creating clusters of sequences that share identical 3'UTRs. Each cluster contains the sequences from a unique gene, together with information on protein similarities, gene expression, cDNA clones and genomic location. This resource not only includes the metazoan data, but also other organisms, including plants and fungi. This analysis and subsequent protein sequence alignments allow achieving a putative function for HRR-containing cluster. The best metazoan matches of HRR cluster transcripts with RefSeq proteins consisted in four RNA-binding animal proteins: *Caenorhabditis elegans* SUP-12 (NP_001129938.1, 56.2% of identity), *Xenopus laevis* XSEB4R (NP_001082613.1, 53.1% of identity), *Mus musculus* RBM38 (NP_062420.2, 52.1% of identity), *Homo sapiens* RBM38b isoform (NP_906270.1, 52.1% of identity). The corresponding HRR orthologue protein sequences were downloaded from GenBank at NCBI and the final alignment was generated, using the Clustal W analysis (MegaAlign software, LaserGene DNASTAR, version7) (Figure 3.5). Despite of low sequence identities with HRR sequence (56.2 – 52.1%), the results indicated a high sequence homology within the RRM domain, particularly in the consensus sequences RNP2 and RNP1.

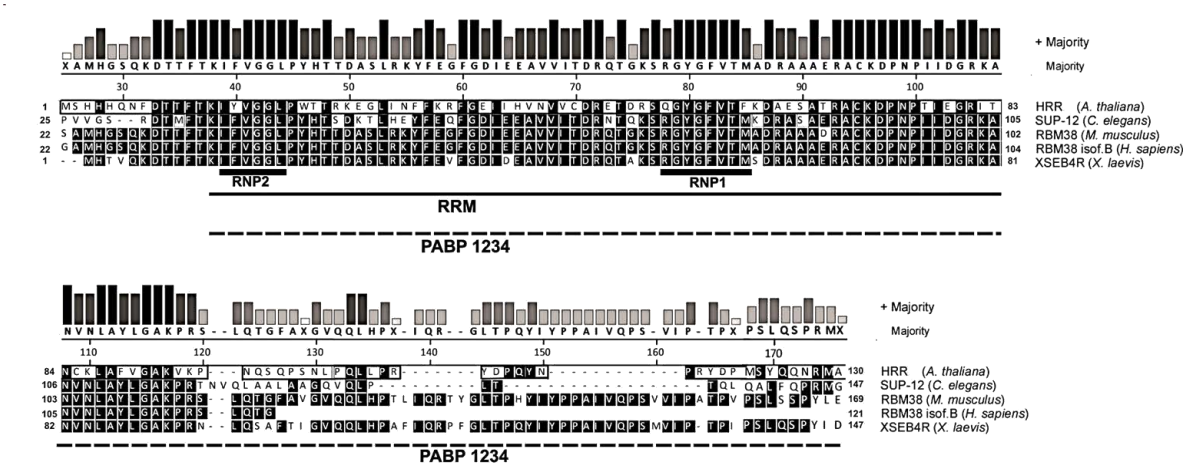


Figure 3.5 Sequence alignment of HRR protein with the most significant orthologues. The amino acid sequences were aligned using MegAlign (Lasergene, DNASTAR v7) with ClustalW method. The five most similar animal proteins used for the alignment were the RRM-containing proteins *Caenorhabditis elegans* SUP-12 (NP_001129938.1), *Mus musculus* RBM38 (NP_062420.2), *Homo sapiens* RBM38b isoform (NP_906270.1) and *Xenopus laevis* XSEB4R (NP_001082613.1).

In contrast to HRR, the other HRR orthologues shared highly conserved sequences in all RRM-domain, even out the RNP2 and RNP1 consensus sequences. In the C-terminal half and spanning the PAPB-1234 domain, only the *Xenopus* XSEB4R (a XSEB4 isoform) and rat RMB38 share a high sequence homology with each other (Fetka *et al.* 2000).

SUP-12 has been described as a novel member of tissue-specific alternative splicing regulator in *C. elegans* and shares high sequence homology with SEB4-related proteins (human SEB4 and *Xenopus* XSEB4 and XSEB 4R isoforms) (Anyanful *et al.* 2004). Recently, the metazoan SEB4 proteins have been annotated as being coded by *RBM38* gene and consequently are known as RBM38-related proteins (NCBI). Due to this high similarity, Anyanful *et al.* (2004) proposed that SUP-12 protein, SEB4 or RBM38-related proteins would be included into a new family of tissue-specific splicing factors of multicellular organisms. The authors also suggested that the corresponding Arabidopsis orthologues could be considered as belonging to the family of AtSEB4a-f-like proteins. Making a brief analysis of their nucleotide sequences (NCBI and TAIR), the results demonstrated that all of them correspond to the best HRR homologues in Arabidopsis, including HRR itself (SEB4-like, e isoform). Another function was described to a metazoan member of this family, *Xenopus* XSEB4R (Souopgui *et al.* 2008). The direct binding of XSEB4R to the 3'UTR region of *VegT* transcripts promotes the increase of their stabilisation and translation. In addition, RNPC1 (annotated as the human RBM-38) has been indicated as the target of p53 tumor supressor, being necessary for maintaining the stability of basal and stress-induced *p21* (a cyclin-dependent kinase inhibitor) transcripts (Shu *et al.* 2006). This stability is promoted by the binding of RNPC1 to the 3'UTR of *p21* transcripts. Since HRR could be considered as a SEB4 like protein, a functional role on splicing or in stabilisation of primary and mature transcripts could be predicted.

Another alignment analysis was performed between HRR protein sequence, its metazoan orthologues and all Arabidopsis RRM-containing proteins, which are distributed among different functional groups: PABPs (poly-A-binding proteins), GR-RBPs (glycine-rich RNA-binding proteins), oligouridylate-binding proteins, snRNPs (small nuclear ribonucleoproteins) and SR proteins (serine-arginine-rich) (Lorkovic and Barta 2002). The final phylogenetic tree was obtained through the sequence alignment using the Clustal W analysis (MegaAlign software, LaserGene DNASTAR, version7) (Figure 3.6). The result confirms the close proximity between HRR and its corresponding orthologues, since all the proteins are clustered together in a single clade. In addition, this HRR metazoan clade is more phylogenetically close to Arabidopsis GR-RBP and oligouridylate-binding protein groups than to PABP, SnRNP and SR protein groups.

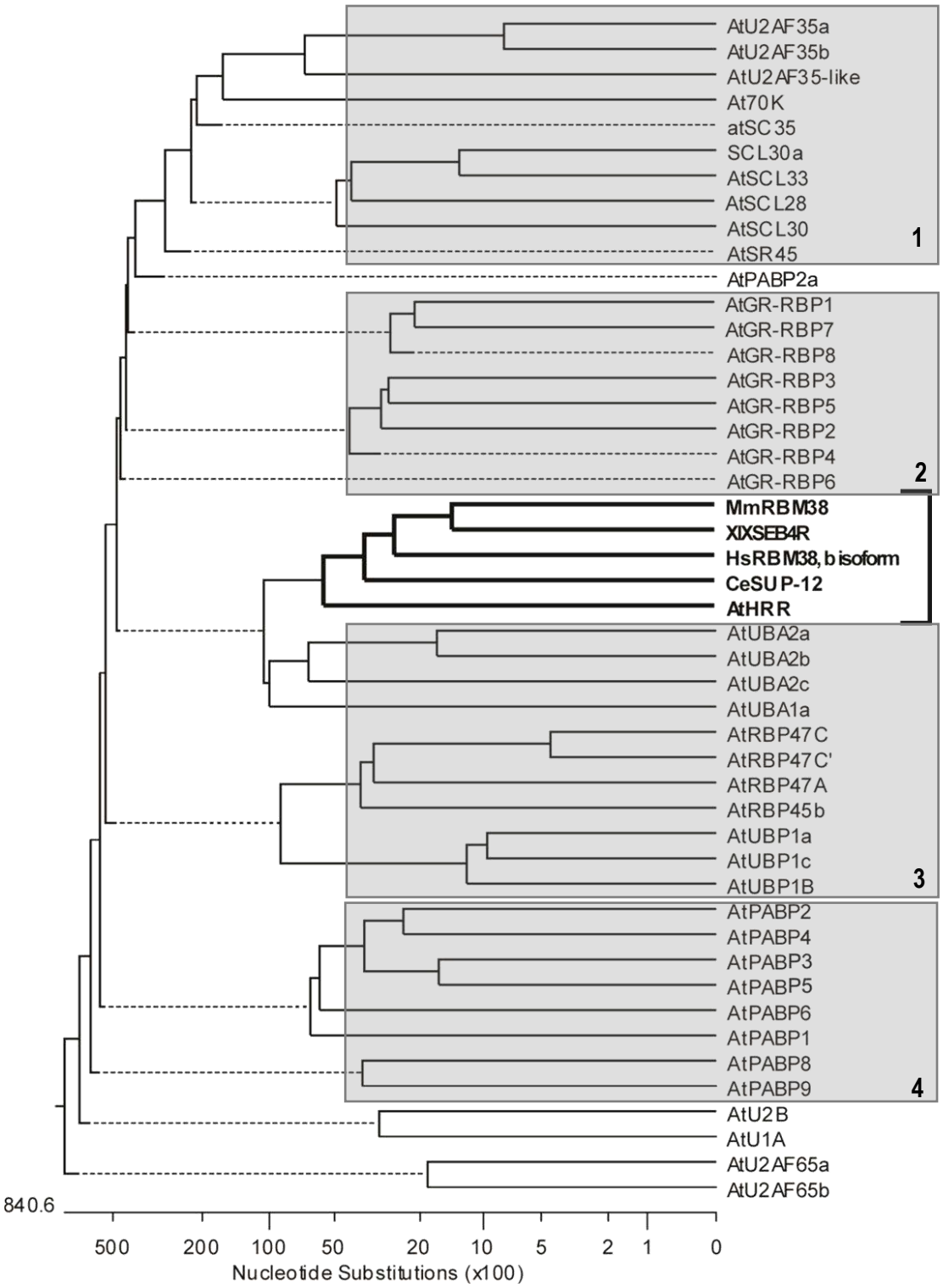


Figure 3.6 Phylogenetic analysis of RRM-containing proteins. The phylogenetic relationship of Arabidopsis RRM-containing proteins and animal orthologues was based on amino acid sequence comparison (Clustal W analysis), using MegAlign (Lasergene, DNASTAR v7). Analysed RRM-containing proteins were selected according to the functional group they belong: 1, snRNP and serine-rich (SR) proteins; 2, glycine-rich (GR) RNA-binding proteins (GR-RBPs); 3, oligouridylate-binding proteins; 4, poly(A)-binding proteins (PABPs). The cluster comprising HRR is distinguished by bold lettering and bracket. The accession gene code for each proteins is depicted in Annex I.

GR-RBPs have been implicated in plant responses to environmental stresses (cold, salt and dehydration) (Lorkovic 2009). Recent studies demonstrated that Arabidopsis GR-RBP7 is required for the efficient export of mature mRNAs from nucleus to the cytoplasm and is highly expressed in cold stress conditions (Kim *et al.* 2008b). Furthermore, this protein was also suggested to act as an assistant in the establishment of better RNA conformations, to turn transcripts more functionally favourable for translation under cold conditions. Other Arabidopsis GR-RBP (GR-RBP2) was equally implicated in responses to cold stress and suggested to have a RNA chaperone activity (Kim *et al.* 2007). In this work, a cold sensitive *Escherichia coli* mutant was complemented on the cold adaptation process by the presence of GR-RBP2 protein. This mitochondrial protein exerts its role by binding mitochondrial targeted transcripts and regulating their processing and/or translation, thus modulating the protein synthesis during cold acclimatisation (Jiang *et al.* 1997; Vermel *et al.* 2002). Another GR-RBP (*Oryza sativa* GR-RBP4) is suggested to promote plant thermotolerance by binding and stabilising the stress-inducible transcripts under HS conditions (Sahi *et al.* 2007).

The group of oligouridylate-binding proteins participe in nuclear protein complexes involved in the recognition of U-rich sequences present in 3'UTRs of mature transcripts. UBA2 and UBA1 isoform proteins have strong binding preferences for oligouridylate sequences in 3'UTRs (Lambermon *et al.* 2002). In addition, UBA2 and UBA1 mediate independent RNA-binding interactions with UBP1, to which they share some similarities. However, in contrast to UBP1, when UBA1 or UBA2 are over-expressed there is a strong accumulation of free poly(A)-mRNAs (Lambermon *et al.* 2000; Lambermon *et al.* 2002). In light of these observations, it was suggested that UBA1 and UBA2 proteins could participate in the composition of different protein complexes (Lambermon *et al.* 2002).

The closer phylogenetic relationship between HRR and GR-RBPs and oligouridylate-binding proteins could suggest a related functional role for HRR: as a transcript stabilising protein and/or as an interaction factor within protein complexes. Playing a role in transcript stabilisation and/or remodelation of RNA metabolism pathways, HRR could indirectly affect the stress-induced proteome, during HS imposition. Indeed the close phylogenetic relationship of HRR with oligoridylate-binding proteins (UBP1, UBAs, RBP45 and RBP47) suggests a regulatory role of HRR in the recognition of U-rich sequences present in 3'UTRs of mature transcripts, being a component of a protein complex.

The HRR-containing clade is more phylogenetically distant from Arabidopsis PABPs group. Searching for conserved domains in some Arabidopsis PABPs (*Conserved Domains* tool, NCBI), the results show that they share homology with metazoan PABP-1234-containing proteins, as referred for HRR (results not shown.) As the Arabidopsis PABPs function has been only predicted based on

their similarity with metazoan counterparts (Lorkovic and Barta 2002), this could suggest that HRR could be also considered as a PABP protein. Nevertheless, a functional divergence should occur due to the presence of only one RRM domain in HRR, in contrast with the four present in Arabidopsis PABP. Ultimately, this could affect the RNA-protein and/or protein-protein interactions However, the possibility of protein interactions between HRR and Arabidopsis PABPs should not be eliminated.

3.1.2 *HRR* promoter analysis

Regarding that HRR is a RNA-binding protein highly up-regulated during HS imposition, a search for promoter *cis*-acting elements was performed. The predicted promoter sequences for *HRR* were obtained through *AGRIS* (*AtcisDB* platform, http://arabidopsis.med.ohio-state.edu/) and Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) databases (Table 3.1). The relevant presence of RAV1 motifs (three consensus sequences) suggests that *HRR* expression is regulated by the binding of RAV1. These transcription factors were initially identified by homology to the B3 domain of maize VP1 transcription factor. Besides this B3 domain (C-termini), RAV1 transcription factor also contains an AP2 domain (N-termini). Therefore, RAV1 binds in a sequence-specific manner to bipartite sequence motifs containing the consensus sequence elements for both the AP2

Table 3.1 Representation of the regulatory regions of *HRR* promoter, using the bioinformatic tools *AtcisDB* (AGRIS) and Athena. Detailed information of promoter binding sites (BS) and corresponding putative TFs that would bind to it are described.

	BS name	Position (start)	BS sequence	BS-associated TFs
AGRIS (AtcisDB)	**ATB2/AtbZIP53/AtbZIP44/GBF5 BS in ProDH**	21815125	ACTCAT	**bZIP**
	W-box promoter motif	21815305	TTGACT	**WRKY**
	CCA1 binding site motif	21815558	AACAATCT	**MYB-related**
	MYB binding site promoter	21814961	AACCAAAC	**MYB**
	MYB4 binding site motif	21814961	ACCAAAC	**MYB**
	RAV1 binding site motif	21814979	CAACA	**ABI3/VP1**
	RAV1 binding site motif	21815481	CAACA	**ABI3/VP1**
	RAV1 binding site motif	21815076	CAACA	**ABI3/VP1**
	BoxII promoter motif	21815578	GGTTAA	**….**
	GATA promoter motif	21815025	AGATAA	**….**
	GATA promoter motif	21815463	AGATAA	**….**
	Ibox promoter motif	21815026	GATAAG	**….**
Athena	**BoxII promoter motif**	-29	GGTTAA	**….**
	CCA1 binding site motif	-49	AACAATCT	**MYB-related**
	Ibox promoter motif	-582	GATAAG	**…**
	MYB binding site promoter	-646	AACCAAAC	**MYB**
	MYB4 binding site motif	-646	ACCAAAC	**MYB**
	W-box promoter motif	-303	TTGACT	**WRKY**

and B3 domains (Kagaya *et al.* 1999). The super-family of B3 transcription factors comprises those that have been described to be involved in seed development and ABA-responsive expression, such ABI3, LEC2 and FUS3 (Giraudat *et al.* 1992; Luerssen *et al.* 1998; Stone *et al.* 2001). Recently, RAV1 transcription factor has also been implicated as a cold-responsive factor and a negative regulatory component of growth development (Hu *et al.* 2004; Yamasaki *et al.* 2004).

Due to the presence of *cis*-acting sequences for binding the AP2 domain (RAV1 motif), it is likely that transcription factors such as ERF, EREBP/AP2, DREB, CBF/AP2 and ABI4 could bind to the *HRR* promoter sequence. These factors are involved in many aspects of plant development, responses to abiotic and biotic stresses, hormonal and metabolism regulation (Finkelstein *et al.* 1998; Liu *et al.* 1998; Kizis *et al.* 2001; Sakuma *et al.* 2002; Gutterson and Reuber 2004; Bossi *et al.* 2009; Hinz *et al.* 2010; Yang *et al.* 2011). The binding of these factors might be mediated through interaction with other transcription factors in a protein complex**.**

Besides RAV1 factors**,** MYB-related and other MYB transcription factors seem to bind to the predicted *HRR* promoter sequence. The MYB transcription factors are involved in many aspects of plant development and metabolism, as well as in responses to abiotic stresses, mainly drought stress (Stracke *et al.* 2001; Abe *et al.* 2003). The presence of a binding site for CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) transcription factor indicates that HRR could be induced during the regulation of circadian rythms. This transcription factor has been indicated as a key element in the transcriptional regulation during the phytochrome signal transduction pathway. The CCA1 transcription factor is greatly related to LATE ELONGATED HYPOCOTYL (LHY), since both harbour a unique MYB domain, so being considered as MYB-related transcription factors (Wang *et al.* 1997). Both CCA1 and LHY have been reported as being involved in regulation of Arabidopsis circadian rhythms, acting in a synergistic mode (Wang *et al.* 1997; Alabadí *et al.* 2002; Lu *et al.* 2009).

The *cis*-elements present in the predicted *HRR* promoter for binding of bZIP transcription factors are *ProDH* promoter-like. These transcription factors belong to the S group of bZIP superfamily and have been associated to sugar and amino acid metabolism, to mid and late stages of seed maturation and to the responses to hypoosmotic and cold conditions (Satoh *et al.* 2004; Hanson *et al.* 2008; Alonso *et al.* 2009; Weltmeier *et al.* 2009; Ma *et al.* 2011). The presence of a W-box in the *HRR* promoter indicates the binding of WRKY transcription factors. Recent data suggest that WRKY factors play key roles in regulation of biotic and abiotic stresses responses, besides being involved in many other physiological aspects, such as embryogenesis and seed coat development, trichome development and regulation of metabolism and hormone signalling (Eulgem *et al.* 2000; Agarwal *et al.* 2011). Recent reports have also referred the involvement of WRKY factors

in responses to heat stress (Li *et al.* 2009; Li *et al.* 2010; Li *et al.* 2011b). The association of bZIP and WRKY transcription factors to plant responses to stress conditions is a good indication that *HRR* promoter could be one target of stress-related gene expression.

3.1.3 *HRR* expression analysis

Being *HRR* expression suggested to be regulated by different types of transcription factors that have been implicated in different developmental and environmental conditions, it is expected that those transcription factors can activate the expression of other stress-related genes. The co-expression of *HRR* was analysed, performing a search in GeneMANIA and ATTED-II platforms. The majority of *HRR* co-expression relationships were predicted by GeneMANIA (Figure 3.7). This platform uses the data obtained from the functional relationships of orthologues that is often related to protein interactions studies, as well as the co-expression, co-localisation and physical interaction data already documented. It is noticeable the predicted HRR co-expression/interaction with other well-known oligouridylate-binding proteins (AtRBP45C, UBP1A and UBP1B and RBP45B). As referred,

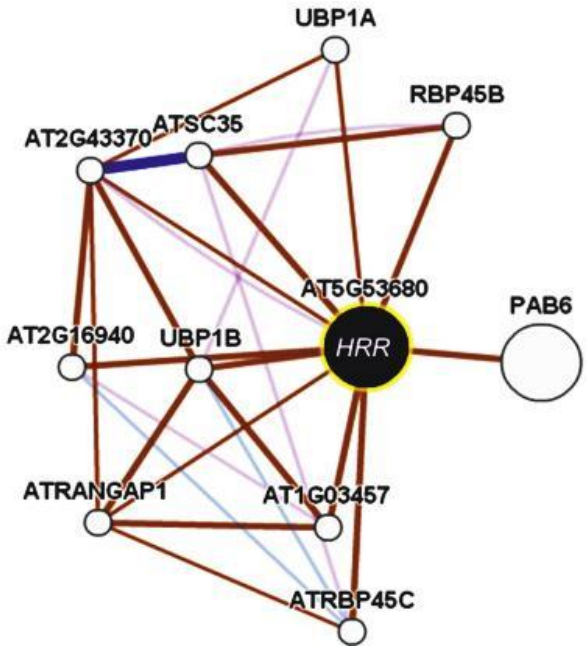


Figure 3.7 *HRR* co-expression network. The gene of interest is black shaded. Brown lines represent the predicted co-expression/interaction relations. Light pink lines represent the co-expression relationships already documented. The co-localised expressions are represented by light blue lines and physical interactions are highlighted by a blue/purple line. Stronger relationships are represented by thicker lines. The genes that are only represented by AGI number do not have an identified function. Genes displaying a higher score are represented by enlarged circles, meaning that they have higher probability of belonging to the networks assigned by the program. Analysis was performed in GeneMANIA platform.

these hnRNP-like proteins are involved in multiple steps of mRNA maturation, from enhancing intron excision to pre-mRNA splicing or mature mRNA stability (Lambermon *et al.* 2000; Lorkovic *et al.* 2000; Lambermon *et al.* 2002). This prediction reinforces the previously suggested genetic and protein interactions of HRR with these U-rich binding proteins.

Also, HRR seems also to be co-expressed or interacting with other RRM-containing proteins, which are still functionally uncharacterised. *At2g16940* codes for a RRM-containing protein putatively involved in mRNA processing. This protein has been annotated as a CC1-like splicing factor and was suggested to be involved in spliceosome pre-assembling (Seraphin 1989; Ascencio-Ibáñez *et al.* 2008). Other co-expressed gene (*At2g43370*) codes for an U1 snRNP-like protein. This protein is also involved in spliceosome assembling and mRNA processing mechanisms (Lorković *et al.* 2005; Schindler *et al.* 2008). *At1g03457* is an orthologue of a *D. melanogaster* gene that codes for a RNA-binding protein described as being involved in mRNA 3'UTR binding (Wang *et al.* 2008; Zhou *et al.* 2010; Peal *et al.* 2011). Considering these predicted co-expressed or interacting data, HRR might also interact with many factors involved in spliceosome assembly and mRNA processing process. Hence, HRR could promote stability to nascent mRNA molecules during the pre-mRNA processing steps.

An interesting aspect is the possible HRR co-expression/interaction with *AtSC35* and *AtRanGAP1*, as well as *PAB6* (or *PABP6*) genes. The AtSC35 is considered as an orthologue of the human splicing factor SC35 and belongs to the family of proteins containing a C- or both terminal domains rich in serine-arginine (SR) dipeptides that could be reversibly phosphorylated/desphosphorylated (Lopato *et al.* 2002; Barta *et al.* 2008). AtSC35 has been described to interact with other SR proteins, particularly with AtRSZ33 phosphoprotein, which seems to play an important role in selection of alternative splice sites and spliceosome assembly (Graveley 2000; Lopato *et al.* 2002). However, the correct selection of splice sites in plants could be promoted by a different set of snRNPs and specific U-rich binding factors, since the plant introns have a high AU content and short U-rich stretches that are required for efficient intron removal (Gniadkowski *et al.* 1996; Brown and Simpson 1998; Barta *et al.* 2008a). In that way, oligouridylate-binding factors, such as UBP or UBA proteins, could participate in intron recognition and their splicing. Indeed, the over-expression of UBP1 enhances the splicing efficiency of the intronic sequences (Lambermon *et al.* 2000). In Arabidopsis, AtRanGAP1 coded by *PARLL-1* was recently annotated as Nucleolin-like 2. This nucleolar protein is suggested to be implicated in ribosomal biogenesis, nuclear signal recognition and nucleocytoplasmic transport (Jeong *et al.* 2005; Petricka and Nelson 2007). PAB6, which presents the highest score for HRR, corresponds to a poly(A)-binding protein, functionally annotated as translation initiation factor (TAIR). Recent data has indicated that *PAB6* is up-regulated during the pollen tube growth, a developmental stage where translation is very active (Wang *et al.* 2008).

All these co-expression/interaction predictions suggest that HRR could play functions in regulation of spliceossome assembly, as well as to be involved in intron recognition and enhancing intron splicing. Moreover, considering the predicted interaction with AtRanGAP1 and PABP6, HRR could be also involved in ribosomal biogenesis, nucleocytoplasmic transport and translation initiation process. These functional previsions for HRR are in accordance with the predicted subcellular localisation of HRR in the nucleus and cytoplasm (Figure 3.8).

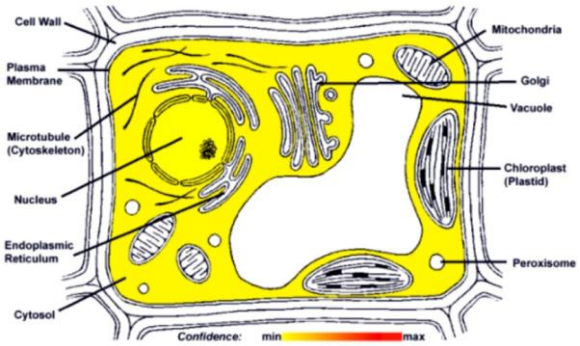


Figure 3.8 Subcellular localisation pattern of HRR. Prediction was performed in *Cell eFP* tool (provided by BAR website). The data indicate that HRR would be located in cytosol and nucleus. These results were obtained through LOCtree, Wolfpsort and Subloc prediction algoritms. Expression levels are identified in colours as depicted in the scale.

The ATTED-II prediction of co-expressed genes with *HRR* is different from the one made by GeneMANIA. The ATTED-II results predicted that *HRR* is co-expressed with *NMT2* (Pearson's correlation coefficient, COR=0.76), *SAP10* (COR=0.74), *FTSH6* (COR=0.73), *AT-HSFA7B* (COR=0.72) and with a transposable element product (COR=0.66). All these genes are associated to stress responses, except the transposable element product with an unknown function. *NMT2* codes for a N-myristoyltransferase, which is involved in addition of the saturated C:14 fatty acid myristate to proteins. This irreversible modification alters the binding properties of crucial cytoplasmic proteins from signal transduction cascades, such calcium-dependent protein kinases and small GTPases (Boisson *et al.* 2003; Pierre *et al.* 2007). The recently annotated *SAP10* (stress-associated protein 10) codes for an AN1-like zinc-finger protein and seems to be involved in responses to toxic metals and high temperatures responses (Dixit and Dhankher 2011). *FTSH6* product corresponds to a chloroplastidial metalloprotease protein that promotes the degradation of Lhcb3 and Lhcb1 proteins from the light-harvesting complex of photosystem II, during senescence and high-light acclimation (Zelisko *et al.* 2005; Wagner *et al.* 2011). The coding gene for the heat shock transcription factor A7B (HSFA7B) is up-regulated under high light and HS conditions. The promoter of this gene is one of the master targets of constitutive HsfA1d and HsfA1e factors. The expression

of HsfA7B thus promotes the extension of thermotolerance responses by up-regulating other heat stress-related proteins (Nosaka *et al.* 2011).

The *HRR* co-expressed genes (predicted by ATTED-II) indicate a complex network of molecular events that occur in early responses to heat stress. The post-translational modifications, promoted in part by N-myristoyltransferases, could change the membrane lipid composition and fluidity that would affect the activity of key kinase proteins. This would lead to alterations in signalling transduction pathways that could affect transcriptional (expression and activity of HSFs and HS-related proteins, such HRR) and post-transcriptional (mRNA splicing, export and stability) and translational mechanisms.

The prediction of *HRR* expression profile was performed using the Bio-Array Resource (BAR) and Genevestigator platforms. Through *Arabidopsis eFP Browser* (BAR), *Metaprofile* (Genevestigator) and *Clustering* (Genevestigator) analysis tools, the expression prediction data was determined in different tissues and organs of Arabidopsis plant (Figures 3.9), during the developmental stages (Figure 3.10), in response to different stress situations and mutant genotypes (Figure 3.11).

The analysis performed in BAR platform for the wild-type Arabidopsis plants, grown under standard conditions, revealed that *HRR* is predicted to be more expressed in mature pollen, early flower bud (stage 9) and final stage of seed maturation (stage 10), exhibiting absolute values of 27.06, 20.41 and 19.51, respectively (Figure 3.9A). Lower expression was predicted for the previous stage of developing seeds (stage 9) (13.63), senescing leaves (10.81), rosette leaves 2 (10.33) and cauline leaves (10.2). All other organs/tissues presented predicted expression levels less than 10.

During seed development, *HRR* is highly expressed mainly in the embryo of in pre- and globular stages, mainly in embryo (22.45 and 31.68, respectively), but also in the corresponding peripheral endosperm tissues (25.96 and 17.69, respectively) (Figure 3.9B). High expression levels were also predicted for general seed coat at globular (19.72), linear-cotyledon (22.76) and mature green stages (17.68). During the initial hours of seed imbibition, *HRR* is expressed at low levels (5.39), achieving higher expression after 24h of imbibition (Figure 3.9C). In experiments where the seeds were further imbibited up to 96h, at 22ºC or 4ºC, the expression levels were much more intense (13.73 and 15.14, respectively). Analysing *HRR* expression in mutant seeds, it is noticeable the higher *HRR* expression in mutant seeds of ABA-related genes, namely *cts1* (19.2) and *fus3-8* (19.05), but also *abi3-4* (14.34). Taken together these results suggest that, under standard growth conditions, *HRR* could play a role during seed development and germination, simultaneously with ABA levels that seem to be crucial for regulation of these development stages.

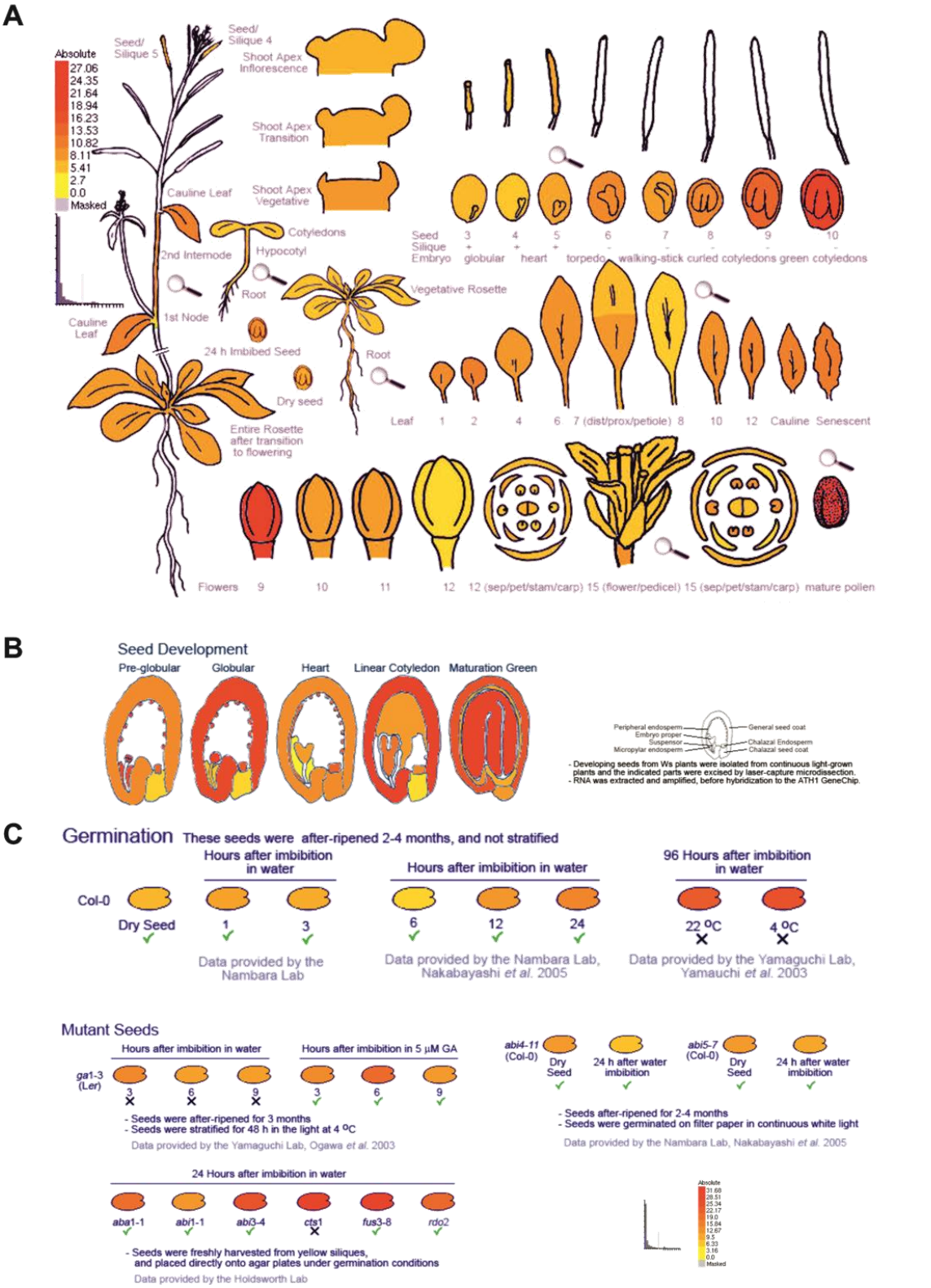


Figure 3.9 Prediction of *HRR* expression in (A) different developmental stages, (B) seed development and (C) germination of wild-type and mutant seeds. *HRR* is highly expressed in mature pollen, early flower bud and final stage of seed maturation. During seed development, *HRR* is predicted to be highly expressed in globular, torpedo and cotyledon stages, being also expressed in imbibed seeds. Some ABA related mutant seeds present higher *HRR* expression levels than wild-type. Expression levels (absolute values) are identified in colors as depicted in the scale. This analysis was performed using Arabidopsis eFP Browser provided by BAR, considering the Development Map (A) and Seed (B,C) data. Seeds that not complete germination are marked with X symbol.

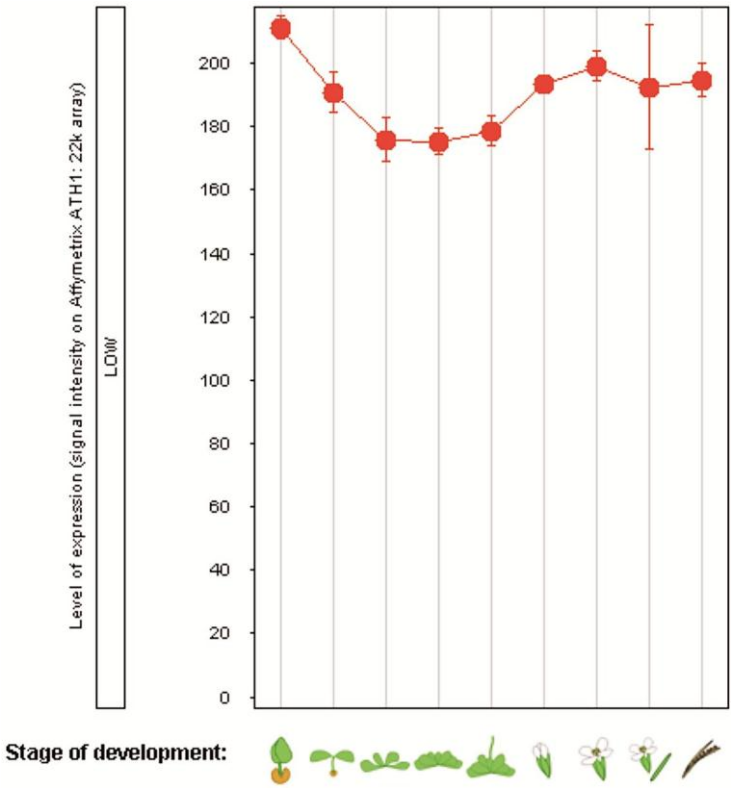


Figure 3.10 *HRR* expression profile during the different stages of Arabidopsis development. Although presenting low levels of expression, the highest levels are observed during seed germination. This analysis was performed considering the signal intensity definitions of ATH1 microarrays. Development stages (from left to right): seed germination, seedling, early rosette, mature rosette, stem formation, early flowering, mature flowering, seed development, desiccation.

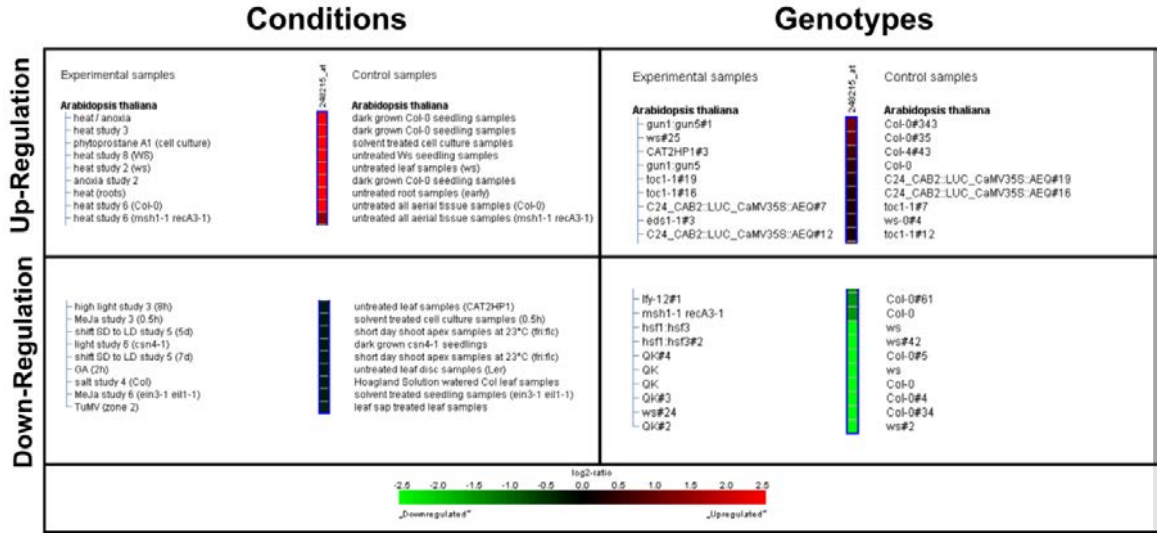


Figure 3.11. *HRR* expression profile in response to different stress situations (conditions) and in different mutant genotypes (genotypes). *HRR* is up-regulated under HS conditions, being also expressed under anoxia conditions. The treatment with phytoprostane A1 promotes the up-regulation of *HRR*. *HRR* expression is mostly affected in HS-related mutants. Expression analysis data were obtained from Meta-Profile analysis tool (Genevestigator). The values correspond to log2 ratio (treatment/ control ratio), being identified in colors as depicted in the scale.

During the different development stages, *HRR* is expressed in all stages, being the highest levels observed during seed germination. However, but at lower levels, the *HRR* expression is also observed during flower development, seed development and dessication (Figure 3.10)

In the majority of experimental conditions analysed, *HRR* is highly up-regulated under HS conditions (Signal Ratio, SR: 157.45; 43.93; 12.66; 9.32), according to the previous prediction of *HRR* as a HS-responsive gene (Figure 3.11). *HRR* is also up-regulated after phytoprostane A1 application (SR: 98.63). Phytoprostane A1 is a cyclopentenone oxylipin that can be formed via enzymatic jasmonate pathway or by a nonenzymatic pathway dependent of ROS, which can function as a signal for the expression of stress-induced genes (Mueller *et al.* 2008). The presence of phytoprostane A1 could thus trigger the mechanisms of HS response, including the *HRR* expression. Hence, this oxylipin could mimic certain functions of heat- induced membrane phospholipids (such as IP3 and PIP2), acting as secondary messengers to promote the further homeostasis adjustment at elevated temperatures. The highest *HRR* up-regulation was detected in anoxia conditions when a heat stress treatment was applied (SR: 275.25). Even without HS, anoxia conditions promote an induction of *HRR* expression (SR: 12.47). The possible cross-talk between anoxia and HS signalling pathways were already suggested when it was found that anoxia conditions promote the induction of HSPs (Banti *et al.* 2008).

In most mutant plants there are no major differences on *HRR* expression (Figure 3.11). However, *HRR* is extremely affected in HS-related mutants (*QK*, quadruple mutant *HsfA1a,1b,1d,1e* and *hsf1:hsf3* double mutant), being also down-regulated in flower mutants (*lfy-1*, *mads* miRNAs) (Weigel *et al.* 1992; Schwab *et al.* 2006; Banti *et al.* 2008; Shedge *et al.* 2010). These results indicate a specific expression of *HRR* during early phases of response to HS, directly or indirectly induced by HSFA1 factors. Indeed, HSFA1a, 1b, and 1d have been described as redundant master regulators, during early phases of HS response (Liu et al. 2011).

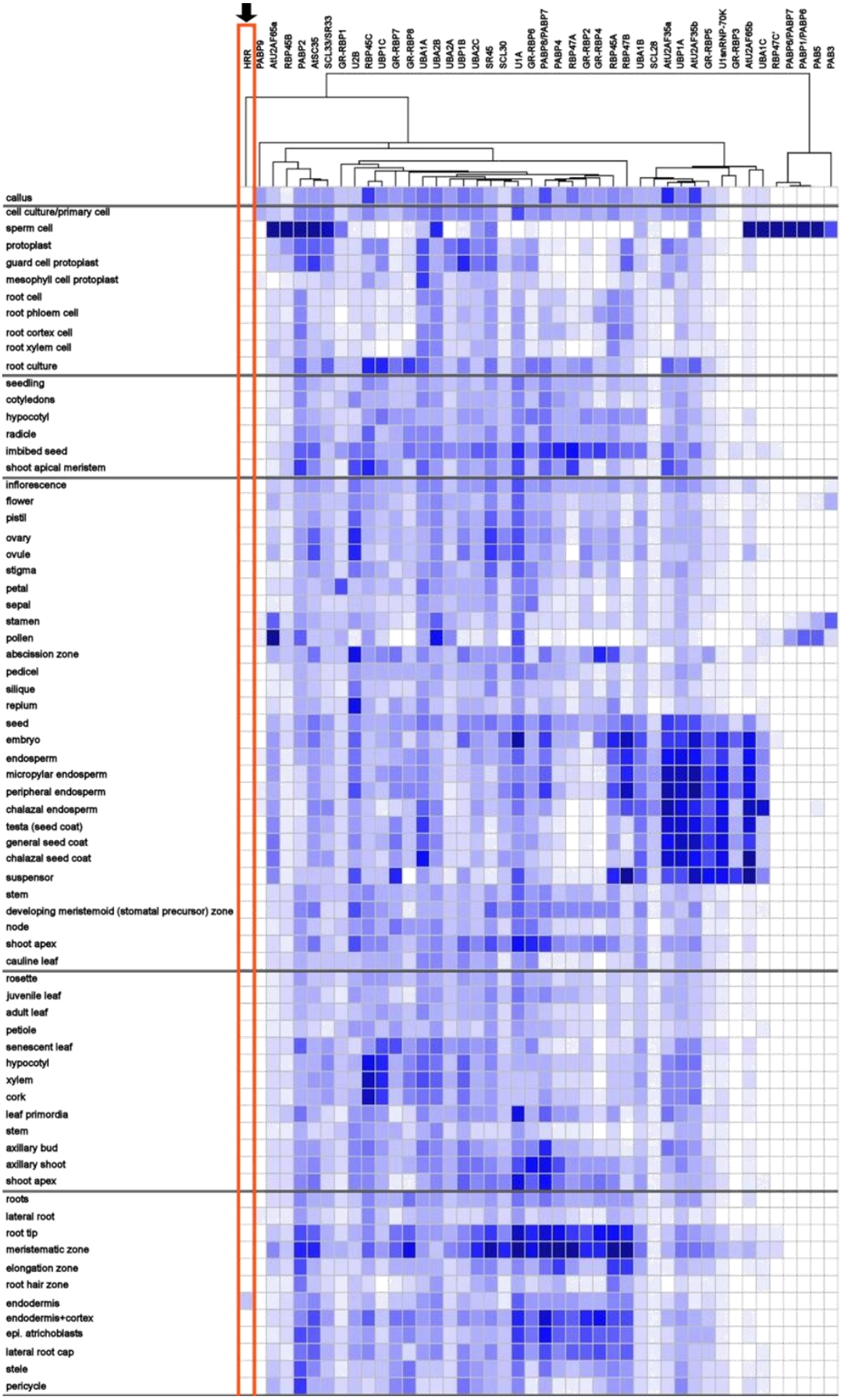
When comparing the *HRR* gene expression with other RRM-containing proteins in different tissues and during plant development, it becomes clear that *HRR* is much less expressed (Figures C3.1 and C3.2). This suggests that the main role of HRR would be on stress conditions. Under different exogenous stimuli, a small number of RRM-containing proteins share expression similarities with *HRR*, namely under HS conditions (Figure C3.3). The phylogenetically close UBA1A presents a very similar expression pattern under HS conditions, but the same expression pattern was also found for the splicing factors SCL33/SR33 and AtSC35, which are more phylogenetic distant from *HRR* (Figure 3.6). In HS experiences performed in *QK* mutants, only *SCL33/SR33* expression levels were as affected as the *HRR* (Figure C3.5). *SCL33/SR33* and *AtSC35* expressions have being described as being affected by HS treatment, generating a different alternative splicing pattern, which in turn

leads to different protein pattern under HS responses (Palusa *et al.* 2007) These results could corroborate the previous predicted functional similarities for HRR and UBA proteins. Once they share some structural and, possibly, some functional similarities as hnRNP-like proteins, it is likely that HRR interacts with UBA proteins during transcriptome regulation, under HS conditions.

Taken together, the *in silico* analysis of *HRR* structure and expression suggests that this RNA-binding protein (a RRM-containing protein) might be an important regulator protein during HS responses. Functional information of Arabidopsis RRM-containing protein homologues and metazoan orthologues suggests that HRR could play a role in the stability of specific HS-induced transcripts, mostly through interaction with other RNA-binding proteins. The phylogenetic relationship with GR-RBPs and oligouridylate-binding proteins suggests that HRR could directly or indirectly bind to HS-induced transcripts, promoting their stability during further RNA metabolism steps. The predicted co-interaction with UBA, UBP and even with spliceossomal factors (AtSC35, SCL33/SR33) also indicates that HRR could bind to certain transcript 3'UTRs, thus preventing their precocious exonucleotidic degradation. In addition, HRR could play auxiliary roles in intron selection and splicing efficiency into spliceossome assembly and activity. Hence, the induction of *HRR* could be important for remodelation of transcriptome during the thermotolerance development. The down-regulation of *HRR* in HS-related mutants (*QK* and *hsf1:hsf3*) (Lohmann *et al* 2004; Liu *et al.* 2011) suggests that HRR could act as an early post-transcription regulator in HS responses. The predicted co-interaction of HRR with RNA export and translation initiation factors (RanGAP and PAB6, respectively) also suggests a nucleocytoplasmic function during developmental or environmental transition of housekeeping transcriptome to HS-specific response transcriptome.

(Right page)Figure C3.1 Gene expression profiles for RRM-containing proteins in different Arabidopsis organs/tissues. The red-lined box corresponds to the *HRR* expression profile. Expression profiles were obtained using the *Clustering analysis* (*Hierarchical Clustering*) for anatomy tool, by Pearson correlation, at the Genevestigator platform (https://www.genevestigator.com/gv/). Results are given as heat maps in blue/white coding (average expression levels), in which blue intensity indicates higher expression level.

3.1.4 Complementary data



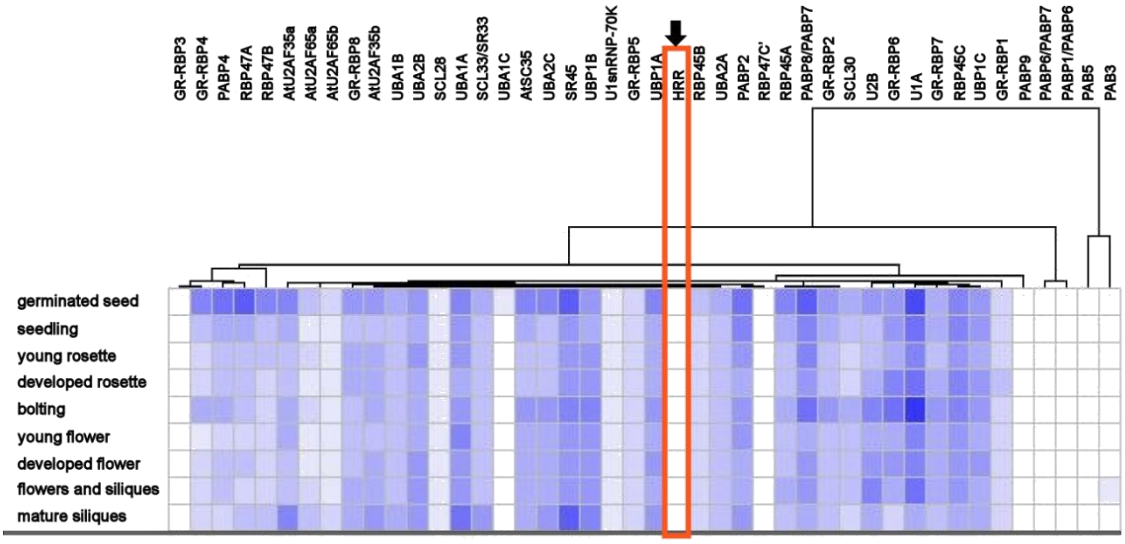


Figure C3.2 Gene expression profiles for RRM-containing proteins during Arabidopsis development. The red--lined box corresponds to the *HRR* expression profile. Expression profiles were obtained using the *Clustering analysis* (*Hierarchical Clustering*) for development tool, by Pearson correlation, at the Genevestigator platform (https://www.genevestigator.com/gv/). Results are given as heat maps in blue/white coding (average expression levels), in which blue intensity indicates higher expression level.

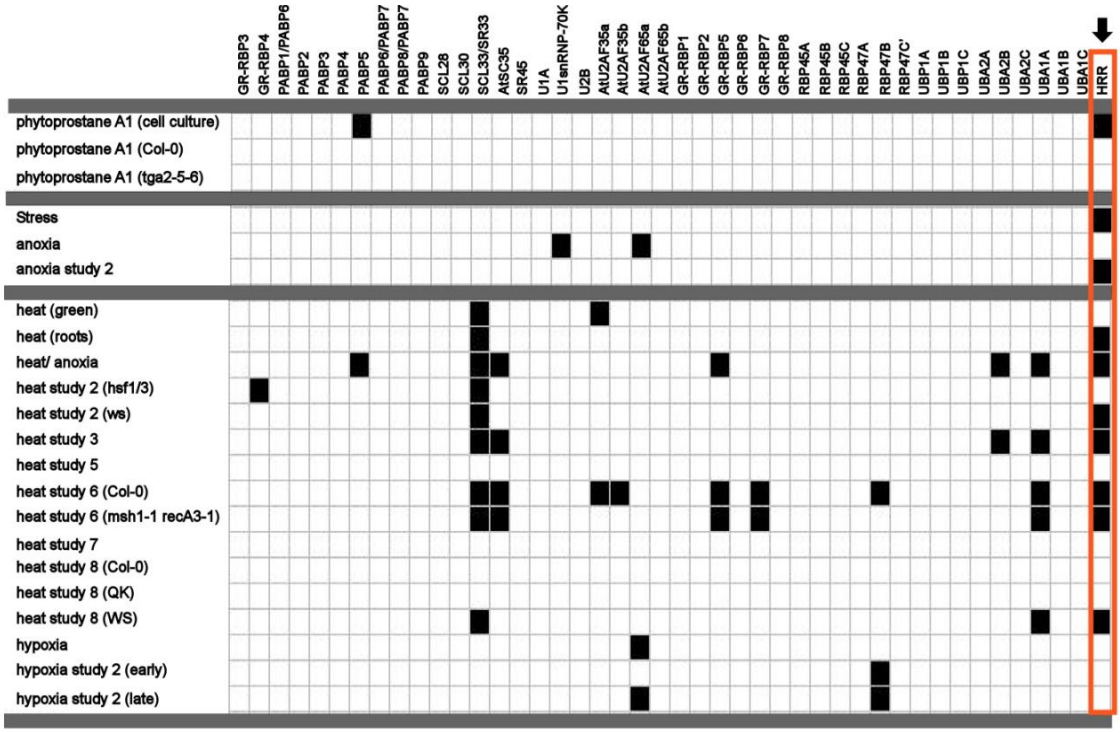


Figure C3.3 Gene expression profiles for RRM-containing proteins in different exogenous stimulus. The red--lined box corresponds to the *HRR* expression profile. Expression profiles were obtained using the *Clustering analysis* (*Biclustering*) for stimulus tool, using BiMax algorithm, at the Genevestigator platform (https://www.genevestigator.com/gv*I*). Results are given as discrete matrix, depicting the up-regulated discretisation values (log2 ratio) between 1.0 and 2.5.

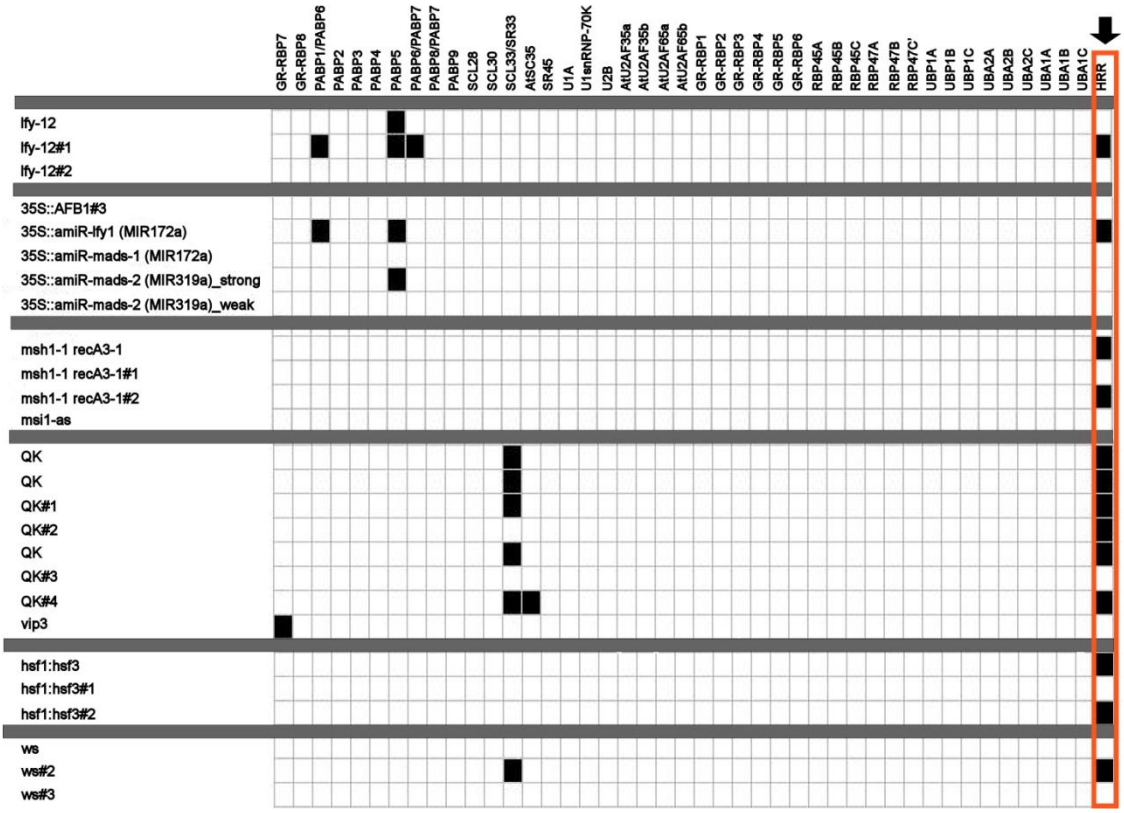


Figure C3.4 Gene expression profiles for RRM-containing proteins in Arabidopsis mutants. The red-lined box corresponds to the *HRR* expression profile. Expression profiles were obtained using the *Clustering analysis* (*Biclustering*) for stimulus tool, using BiMax algorithm, at the Genevestigator platform (https://www.genevestigator.com/gv/). Results are given as discrete matrix, depicting the down-regulated discretisation values (log2 ratio) between -2.5 and -1.0.

3.2 Phenotypic characterisation of *hrr* loss-in-function and HRR over-expression mutant lines

The functional characterisation of *HRR* was initiated by studying the loss-in-function and over-expression lines for *HRR*, both in wild-type *Ler* ecotype background.

3.2.1 Isolation of *hrr* loss-in-function and HRR over-expression lines

A loss-in-function line (GT_5_47364) containing a *Ds3-1* transposon insertion in the third exon of *HRR* (Figure 3.12), was ordered from public stocks (NASC) (Clarke 2000). As the acquired seed pool corresponds to the heterozygous F3 generation, the screening of homozygous for that insertion was initiated. The genomic DNA was extracted from each individual F3 plant and used in a diagnostic PCR analysis. For this, a multiplex PCR method was performed making use of three primers: two gene-specific primers (HRR_LP and HRR_RP) for flanking the *Ds3-1* transposon-insertion and an insertion-specific primer (prbZF_Rv) (Figure 3.12A, Annex III). The wild-type (*Ler*)

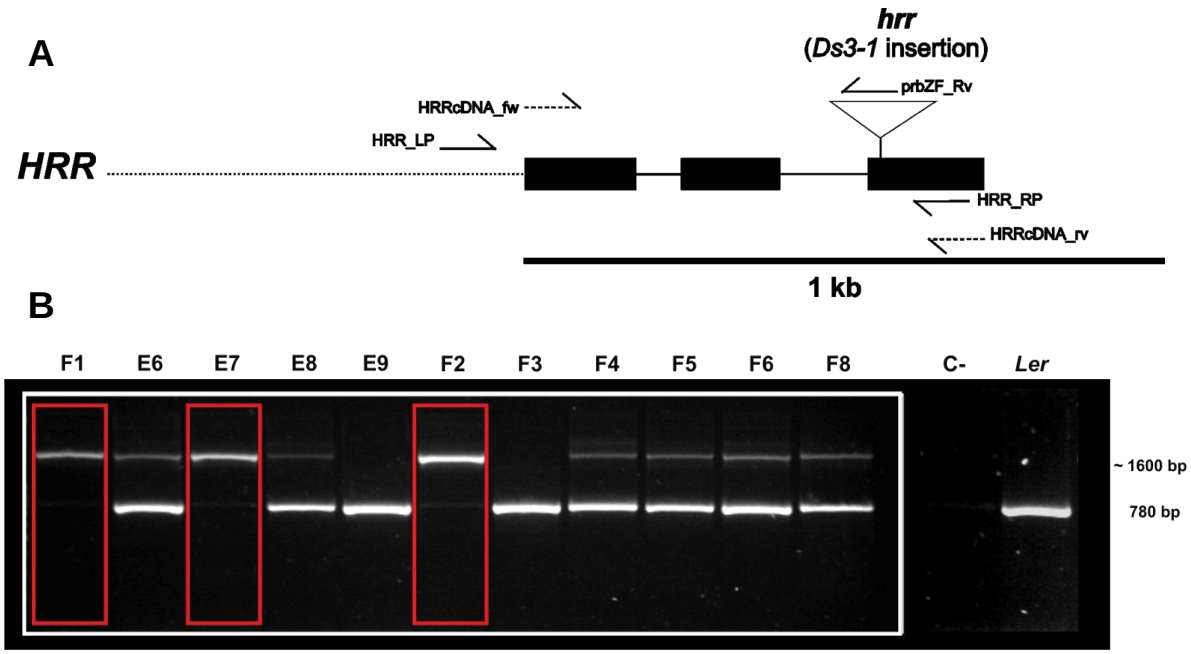


Figure 3.12 Isolation of homozygous *hrr* mutant line. (A) Schematic representation of *HRR* gene depicting a *Ds3-1* transposon-containing insertion (triangle) in the third exon (position 21816179, chromosome 5). The predicted promoter region is represented by a dashed line. The full arrows depict the diagnostic primers used for plant genotyping by PCR. The dashed arrows depict the primers used for *HRR* expression analysis by semi-quantitative RT-PCR. (**B**) Electrophoretic analysis of diagnostic PCR products for genotypic identification of *hrr* mutant line. Public available *HRR Ds3-1* transposon-containing insertion line was ordered and each F3 plant was tested for homozygous insertion-containing individuals through diagnostic PCR. The picture represents a typical analysis, in which 11 F3 plants (F1-F8) were tested. The identified homozygous mutant lines are evidenced by orange boxes. Controls were performed with wild-type (*Ler*) DNA and without any DNA (C-).

genomic DNA was used as a positive control, since does not hold any *Ds3-1* transposon-insertion. In this case, a single PCR product of 780 bp was obtained, resulting from the amplification with both gene-specific primers (Figure 3.12B). As homozygous plants contain *Ds3-1* transposon insertions in both chromosomes, a single PCR product of about 1600 bp was amplified by the insertion-specific (prbZF_Rv) and gene-specific (HRR_LP) primers. In this case, no PCR product will result from amplification using both gene-specific primers. As hemizygous plants only harbour a *Ds3-1* transposon-insertion in one of the chromosomes, the PCR amplification will result in both fragments (780 and ≈ 1600 bp). Three distinct homozygous insertion mutants (F1, E7 and F2) were depicted (Figure 3.12B). The same unspecific amplification was also detected in the negative control (without DNA).

HRR over-expression lines were generated through the Gateway® recombination system (Invitrogen). The complete *HRR* cDNA sequence was inserted into the pMDC83 pDEST vector (Curtis and Grossniklaus 2003) that harbours a strong promoter (*2x35S*) and thus provides an HRR over-expression system. The resulting construct *p35S::HRR-GFP6* (Figure 3.13A) was used to transform *Agrobacterium tumefaciens* (EHA105 strain), which was then used to transform the *hrr* mutant and wild-type *Ler* plants. The selection of transgenic lines was performed by germinating seeds onto selective MS medium (supplemented with hygromicin). In the first, generation T2 from independent HRR over-expression lines were recovered: JP5, JP6 and JP9 (in *hrr* mutant background) and L2 and L6 (in wild-type *Ler* background). For confirming the presence of the transgene construct in the genome, all over-expression lines were subjected to a diagnostic PCR analysis. For this PCR, the primers pMDC35S (for the 35S promoter sequence) and pMDCgfpleft (for the GFP6 coding sequence) were designed for allowing the amplification of all *HRR* coding sequence in the transgene construct (Figure 3.13A, Annex III). Using such a PCR analysis, the previously selected lines were proved to contain the transgene construct in genome (Figure 3.13B).

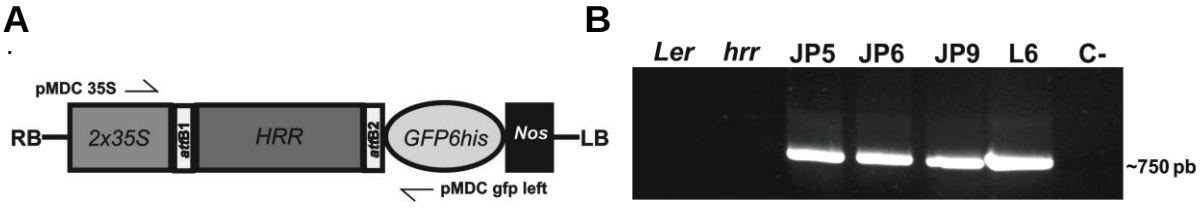


Figure 3.13 Selection of HRR over-expression lines. (A) Schematic representation of the transgene construct *p35S::HRR.1-GFP6*. The arrows depict the diagnostic primers used for plant genotyping by PCR. *2x35S*, dual CaMV *35S* promoter; *att*B1 and *att*B2 recombination sites; *GFP6his*, *GFP6* coding region; *Nos*, *nos* gene terminator; RB and LB, righ and left borders, respectively. (**B**) Electrophoretic analysis of diagnostic PCR products for genotypic identification of *HRR* over-expressing lines. The T4 transgenic plants from each selected HRR over-expression line (JP5, JP6, JP9 and L6) were tested for the transgene presence by diagnostic PCR. Controls were performed with wild-type *Ler*, *hrr* mutant and without (C-) genomic DNA.

Indeed, regarding that *HRR* cDNA sequence possesses 510 bp and considering the position of primers in specific construct elements, the expected size (~750 bp) was obtained for each selected line. As expected, no amplification was detected in wild-type *Ler* and *hrr* mutant (Figure 3.13 B). Each HRR over-expression line was used for seed bulk production, being synchronised with wild-type *Ler* and *hrr* mutant.

3.2.2 *HRR* expression analysis in *hrr* and HRR over-expression mutant lines

To confirm that *hrr* mutant is a knockout line and HRR over-expression lines constitutively produce high amounts of *HRR* transcripts, a *HRR* expression analysis was performed in wild-type *Ler*, *hrr*, and over-expression mutant lines, under standard growth conditions. Total RNA samples from wild-type *Ler* and *hrr* mutant plants were extracted from different Arabidopsis organs/tissues: inflorescences, rosette, cauline leaves (from five-weeks old plants), roots and cotyledons (16-days -old seedlings). In case of HRR over-expression lines, the *HRR* expression analysis was performed in RNA samples taken from 16-days-old seedlings. After synthesis of the corresponding cDNAs, a semi-quantitative RT-PCR was performed using gene-specific primers (depicted in Figure 3.12A, Annex III). In wild-type (*Ler*) samples, *HRR* is more expressed in inflorescences (2), and less expressed in cauline (1) and rosette (3) leaves, as well as in roots (4) (Figure 3.14). In cotyledons (5), *HRR* expression is undetectable. These results are in agreement with eFP *Developmental Map* data, in which *HRR* achieved the highest expression levels during flower development, exhibiting lower levels in cauline and rosette leaves (Figure 3.9A). Genevestigtor data also predicted the expression of *HRR* in roots (Complementary Figure C3.1), which was confirmed by the *HRR* transcript levels found in seedling roots (Figure 3.14). In the same plant tissues, no *HRR* expression was detected in *hrr* mutant lines, supporting the suggestion of *hrr* mutant being a knockout line.

All HRR over-expression transgenic lines were expressing *HRR,* as confirmed by RT-PCR analysis (Figure 3.15). While JP5, JP6 and JP9 transgenic lines exhibit an evident over-expression of *HRR*, the transgenic lines L2 and L6 display low *HRR* transcript levels. As expected, under the same conditions, both wild-type *Ler* and *hrr* mutant seedlings did not express *HRR*. The differences on *HRR* expression among the over-expression lines could be explained by their different genetic backgrounds. The dip transformation of *hrr* mutant and wild-type *Ler* plants with a HRR over-expression construct was followed for analysing the complementation of *hrr* mutant plants with a wild-type *HRR* copy (JP5, JP6 and JP9 lines) and to strength the *HRR* expression (L2 and L6 lines). However, while the ectopic *HRR* expression in *hrr* mutant background is evident, in *Ler* background the *HRR* expression is limited. The occurrence of a RNA silencing phenomenon, mediated by

siRNAs, could be responsible for the low levels of *HRR* transcripts in those lines containing a wild-type background. Once a substantial amount of foreign homologous transcripts is perceived, the production of double-stranded small RNAs (~20-25 nt RNAs) by siRNA biogenesis machinery [*Arabidopsis* DICER-LIKE 2 (DCL2), DCL3 and DCL4] would be triggered. These siRNA will be incorporated in RNA-induced silencing complex (RISC) that would mediate the degradation of complementary mRNAs or even guide chromatin modification and transcription silencing processes (Filipowicz *et al.* 2005; Brodersen and Voinnet 2006; Vazquez 2006; Pontes and Pikaard 2008).

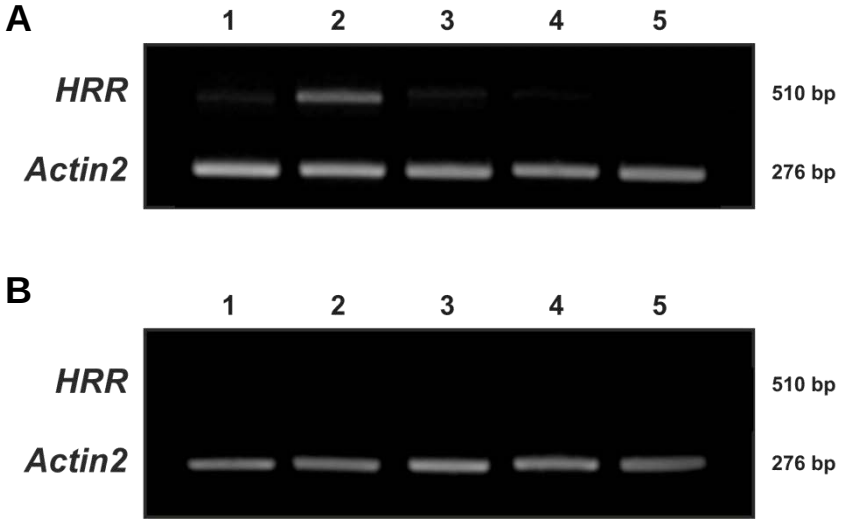


Figure 3.14 Expression analysis of *HRR* in different organ/tissues of Arabidopsis (A) wild-type *Ler* and (B) *hrr* mutant plants. Semi-quantitative RT-PCR was performed from total RNA (1 µg) extracted from cauline leaves (1), inflorescences (2) and rosette leaves (3) of five-weeks-old plants. Expression analysis in roots (4) and cotyledons (5) was determined in 16-days-old seedlings. Wild-type *Ler* and *hrr* mutant plants and seedlings were grown at standard conditions. PCR was performed using HRRcDNA_fw and HRRcDNA_rv primers and 28 cycles of amplification. Numbers on the right refer to the expected sizes of PCR products. As internal control, the transcript levels of *Actin2* (*ACT2*) were analysed.

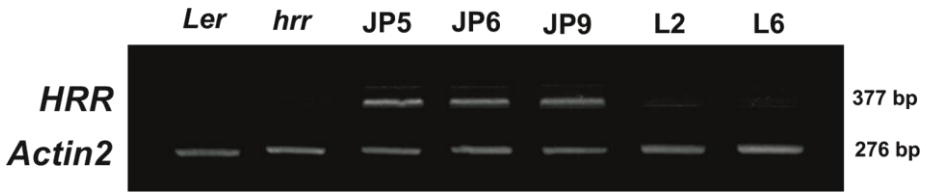


Figure 3.15 Expression analysis of *HRR* transgene in HRR over-expression lines. Transcript levels were determined by semi-quantitative RT-PCR from total RNA (1 µg) extracted from 16-days-old seedlings. Wild-type *Ler*, *hrr* and p*35S::HRR-GFP* lines, established in *hrr* mutant (JP5, JP6 and JP9) and wild-type *Ler* (L2 and L6) plants, were grown under standard conditions (23ºC, control). PCR was performed using HRRcDNA_fw and HRRcDNA_rv primers and 28 cycles of amplification. Numbers on the right refer to the expected sizes of PCR products. As internal control, the transcript levels of *Actin2* (*ACT2*) were analysed.

3.2.3 Seed germination of *hrr* and HRR over-expression mutant lines after a HS treatment

Microarray data indicated that *HRR* expression is induced just after HSS imposition (one to three hours, 38ºC; heat stress experiences in the AtGenExpress *Abiotic Stress Series*) and during seed germination (see in 3.1 section). For predicting the role of *HRR* in Arabidopsis, the germination was evaluated in wild-type *Ler* and *hrr* mutant seeds, under standard conditions or upon a HS treatment. Stratified seeds (4ºC, 2 days) were exposed to different HS treatment periods/temperatures and immediately sown onto MS medium. The germination of both seed lines was affected by raising the periods/temperatures of HS treatment, being completely impaired for treatment periods longer than 180 min at 50ºC and heat treatments above 53ºC for 60 min (Figure 3.16; Complementary Figure C3.5). When a HS treatment of 50ºC was applied during 30 or 60 min, the germination of *hrr* mutant seeds was more affected than *Ler* seeds. Also, a HS treatment at 47ºC and 50ºC, during 60 min, reduces more the germination of *hrr* mutant seeds than wild-type *Ler*. Altogether, the results suggest a thermotolerance phenotype in *hrr* mutant seeds.

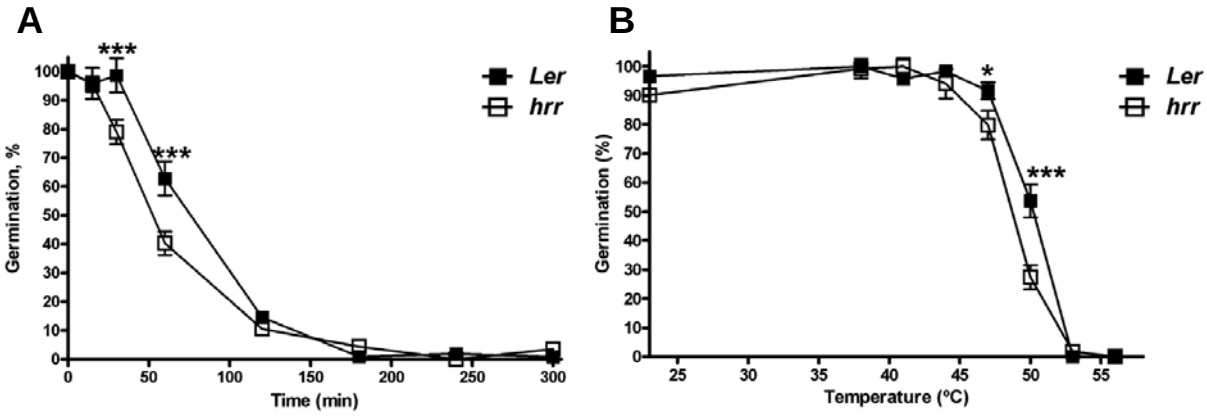


Figure 3.16 Evaluation of heat stress effects on *hrr* mutant seed germination. After stratification (4ºC, 2 days) and before sowing onto MS medium, wild-type *Ler* and *hrr* mutant seeds were **(A)** heat-stressed at 50ºC, for periods ranging from 0 (control) to 300 min or **(B)** heat-stressed for 60 min, at different temperatures, ranging from 23ºC (control) to 56ºC. Seed germination was evaluated by scoring the radicle emergency 10 days after heat treatment. Results obtained from four replicates (30 seeds each) were normalised with corresponding germinated seeds in control conditions. Data correspond to means ± SEM. * and ***, significant differences at P< 0.05 and P<0.001, between *hrr* and *Ler* seed germination, according to the two-way ANOVA (Bonferroni test).

The highest differences between *hrr* mutant and wild-type *Ler* germination occurred between 30 and 60 min of HS treatment, suggesting that HRR is rapidly recruited for early HS responses. The extensive *HRR* down-regulation in *QK* mutants (quadruple mutant *HsfA1a,1b,1d,1e*) after HS treatment (Complementary Figure C3.4) suggests that *HRR* expression could be directly or indirectly

regulated by HSFA1 regulators (Liu *et al* 2011). The family of HSFA1 transcription factors is constitutively expressed, but remains in an inactive conformation under non-stressful conditions, in complex with HSP90/HSP70 chaperones (Schöffl *et al.* 1998). When a heat shock occurs, HSP90 and HSP70 are recruited and these transcription factors became activated. Ultimately, HSFA1s promote the gene expression of HS-related genes, namely those that code for HSPs, other HSFs, as well as other transcription factors (WRKY, MYB, AP2/EREBP) (Busch *et al.* 2005). As HRR seems to be recruited during the first hour of HS treatment, HRR could regulate or interact with these HS-induced transcripts. Being *HRR* promoter sequence enriched in *cis*-elements for binding of WRKY, MYB and AP2 transcription factors (Table 3.1), HSFA1 could indirectly induce *HRR* expression. A recent study demonstrated that some HSFA1-targeted genes (*HSFA2, HSP101, HSP25.3* and *HSA32*) are highly up-regulated after 60 min of HS imposition (Liu *et al.* 2011). Indeed, the HSFA2 factor is later induced by HSFA1 proteins and promotes the expression of *HSP* genes (Li *et al.* 2011a). For this, HSFA2 is considered a key component of the HSF signalling network involved HS responses, function as replacer of HSFA1 factors (Schramm *et al.* 2006; Li *et al.* 2011a; Nosaka *et al.* 2011).

To determine if the observed seed phenotype was really associated with a unique insertion in *HRR* gene and not related with additional gene disruptions, a genetic complementation assay was performed using the HRR over-expression lines. The wild-type *Ler*, *hrr* mutant and HRR over-expression transgenic seeds were subjected to a HS treatment of 50ºC for 60 min, followed by a similar germination assay as described before. The transgenic lines JP5, JP6 and JP9 (in *hrr* mutant background) exhibited the highest germination levels, compared to the wild-type that exhibited similar levels to L2 and L6 over-expression lines (in the wild-type *Ler* background) (Figure 3.17, Complementary Figure C3.6). As expected, *hrr* mutant seeds presented a reduced germination in this condition.

The presence of *HRR* over-expressing transgene in *hrr* mutant (JP5, JP6 and JP9 lines) not only complemented the mutation, but also improved the thermotolerance of corresponding seeds, when compared with those from wild-type *Ler* (Figure 3.17). This suggests that HRR could play a crucial role in thermotolerance of heat-stressed seeds. The lower germination rate of L2 and L6 seeds upon HS treatment, yet not significantly different from wild-type *Ler* could result from reduced levels of *HRR* transcripts and protein in these seeds. This could be due to a possible post-transcriptional silencing mechanism. As a result, HRR could be insufficient to induce seed thermotolerance.

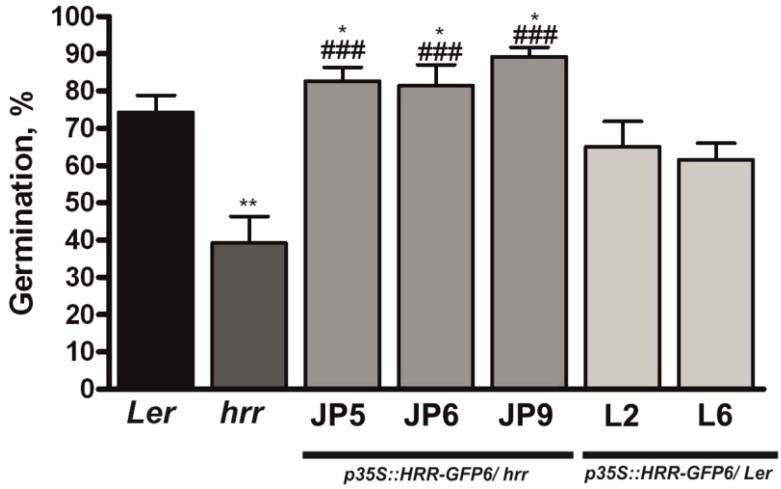


Figure 3.17 HRR over-expression transgenic seeds recover seed germination capacity under heat stress conditions. After stratification (4ºC, 2 days), seeds from wild-type *Ler*, *hrr* mutant and different p*35S::HRR-GFP* transgenic lines (in *hrr* mutant and *Ler* backgrounds) were heat-stressed (50ºC, 60 min) and directly sown onto MS medium. Seed germination was evaluated by scoring the radicle emergency 10 days after heat treatment. Results obtained from four replicates (30 seeds each) were normalised with corresponding germinated seeds in control conditions. Data correspond to means ± SEM. ** and *, significant difference at P<0.01 and P<0.05, when compared with Ler; ###, significant difference at P<0.001, when compared with *hrr* mutant (one-way ANOVA, Tukey test).

The higher *HRR* transcript levels in JP5, JP6 and JP9 (Figure 3.15) could already have predicted the better thermotolerance ability of their seeds. High accumulation of HRR protein, in standard growth conditions, could improve its action during early stages of HS imposition and during the recovery period. As the *HRR* transcript levels are lower in L2 and L6, the response to high temperatures based on HRR function would be weaker and similar to the wild-type *Ler*. Here, even considering a possible post-transcriptional silencing mechanism, it is not excluded the possibility the intervention of other HS-response pathways to built the thermotolerance response in L2 and L6 lines.

Few studies involving RNA-binding proteins in plant thermotolerance processes have been published. The over-accumulation of rice OsGR-RBP4 protein has been reported to be critical for survival of wild-type yeast cells at high temperatures. The respective coding gene has also been reported to be constitutively expressed as well as up-regulated by different stresses, particularly by high temperature stresses (Sahi *et al.* 2007). As this protein comprises an N-terminal RRM domain and a C-terminal sequence rich in arginine and glycine residues, being phylogenetically close to Arabidopsis GR-RBP7, a possible role on the protection of HS-related transcripts by binding to mRNA molecules during elevated temperatures has been suggested. Yet, this protein revealed to be a RNA shuttle protein, between the nucleus and cytoplasm, during HS treatments (Sahi *et al.* 2007). As HRR over-expression confers heat tolerance in germinated seeds, a similar function of HRR in structural stabilisation and protection of transcripts at elevated temperature could be predicted,

namely in the stabilisation of mRNAs from stress-related genes (corresponding transcripts for HSFs, HSPs, sHSPs, LEA proteins and dehydrins).

3.2.4 Phenotypic analysis of *hrr* mutant under salt, osmotic and oxidative stresses

The majority of abiotic stress studies in plants have been focused on single stress treatments applied under controlled conditions. However, field plants are subjected to simultaneous stresses, such as drought, high salinity, extreme temperatures and high UV-irradiance levels, which limit the plant growth and productivity. When combined together, such conditions may induce different gene expression profiles from those obtained in laboratory. Even though *HRR* is mainly expressed under HS treatment, germination assays using *hrr* mutant seeds were performed under different abiotic stresses and their combination with HS. Stratified seeds (4ºC, 2 days) of *hrr* mutant and wild-type *Ler* were sown onto MS agar medium supplemented with different concentrations of stress-inducible agents (NaCl for salt stress, mannitol for osmotic stress and paraquat for oxidative stress). Assays were performed with untreated or with HS-treated seeds (47ºC, 60 min). The percentage of 10-day-old seedlings with fully and green cotyledons (survival rate) was determined (Figure 3.18).

The survival rates of *hrr* mutant and wild-type *Ler* seedlings were compromised in the presence of salt, being completely impaired at salt concentrations higher than 150 mM (Figure 3.18A). Only a significant difference was found for wild-type *Ler* seeds directly sown onto a MS medium with a concentration of 75 mM NaCl. When a combination of heat and salt stresses was imposed, the survival rate of both seedling lines was even more affected than subjecting seeds only to salt stress. Either in combination with HS or not, the survival rate of *hrr* mutant seedlings was always more reduced than in wild-type *Ler*, though there were no significant differences between survival rates of different experimental conditions. A significant difference was detected between salt and salt/HS imposition in both lines for 75 mM and 100 mM NaCl.

In case of osmotic stress treatment, the seedling survival of both lines is completely impaired at the highest concentrations of mannitol (7%, w/v) (Figures 3.18B). Under osmotic stress, there were not significant differences between survival rates of *hrr* mutant and wild-type *Ler.* However, seedling survival rate differences were detected between single abiotic stress and HS-combined abiotic stress imposition. This difference was statistically different in both line for osmotic and HS-osmotic stress combination, at 5% mannitol.

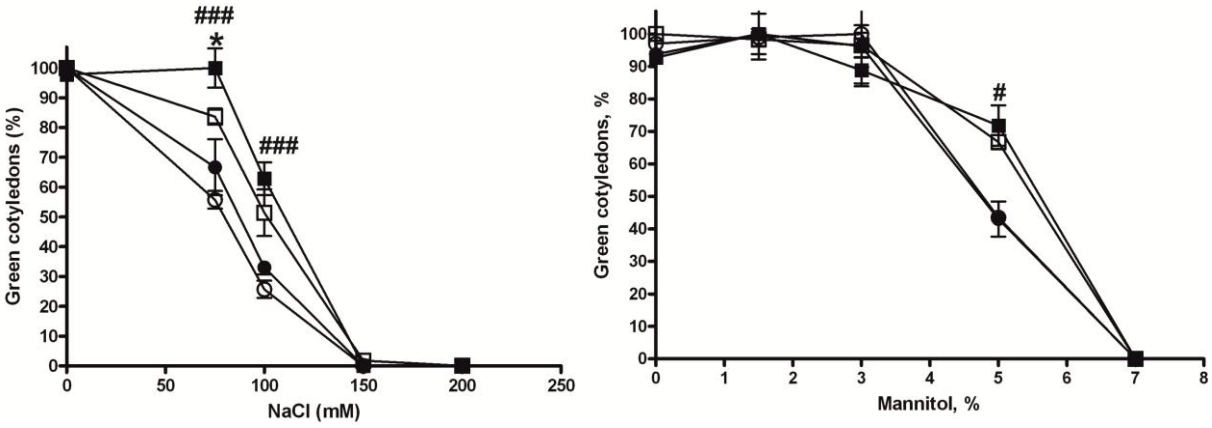


Figure 3.18 Evaluation of the effect of abiotic stresses and their combination with HS treatment on *hrr* mutant. The effect of (A) salt and (B) osmotic stresses was evaluated by plating stratified seeds (4ºC, 2 days) onto MS agar medium supplemented with different concentrations of salt (NaCl) and mannitol, respectively. Assays were performed in wild-type *Ler* (closed symbols, ■ and ●) or *hrr* mutant seeds (open symbols, □ and ○), without HS treatment (square symbols, ■ and □) or previously heat-stressed at 47ºC for 60 min (circle symbols, ● and ○). Viable seedlings, displaying green cotyledons, were scored 10 days after sowing. Results obtained from three replicates (40 seeds each) were normalised with corresponding viable seedlings in control conditions. Data correspond to means ± SEM. *, significant difference at P< 0.5, when compared with *hrr* mutant, in same conditions. ###,, significant difference at P< 0.001 when compared with salt and salt/ HS stresses, in both lines. #, significant differences at P<0.5, when compared with osmotic and osmotic/heat stresses, in both lines; (two-way ANOVA, Bonferroni test).

For performing these phenotypic analyses, stratified seeds were previously heat-stressed and then sown onto each stressful MS-agar medium or were directly sown onto the normal media. Therefore, during the germination and seedling growth, seeds/seedlings were continuously subjected to the other abiotic stress, while they were recovering from HS treatment. These experimental conditions could result in a different response that would be obtained if salt, osmotic or oxidative stress were imposed before HS treatment. In a previous study, where a transcriptome and metabolome analyses were performed during a combination of drought and heat, the HS treatment in Arabidopsis plants was imposed after drought treatment (Rizhsky *et al.* 2004). This difference between experimental designs could be reflected in the obtained results in wild-type *Ler* and *hrr* mutant survival rates observed for each combination of stress conditions, what could mask putative functions of HRR in transcriptome under salt and osmotic stresses.

The highest impairment of *hrr* mutant under salt stress suggests that HRR could be involved in responses to salt stress conditions, under these experimental conditions. A similar situation was observed for *grp2* mutant, whose germination was affected at 75 mM of NaCl (Kim et al 2007). GRP2 is a glycine-rich RNA binding protein that plays important roles in Arabidopsis seed germination under stress conditions. At 100 mM of NaCl, as the difference between wild-type *Ler* and *hrr* mutant was not significant, this could be related with a possible saturation of response ability to extreme salt conditions. In a previous work, Na^+ concentration of 0.1M was cytotoxic, affecting

specific biochemical and physiological processes (Ramón 1996). With a previous HS treatment, both lines are greatly affected, though there was not difference between germination of wild-type *Ler* and *hrr* mutant. These results indicate a cumulative effect of both stresses, possibly implicating HRR in responses to both stresses. Indeed, by little difference of survival rates, it is possible that HRR function could not be sufficient to cope with such extreme conditions, such as occurs at 100 mM. A previous study demonstrated that wheat seedlings treated with a combination of salt (0.7%) and heat (40/30ºC) stresses were drastically affected in shoot elongation (Keleş and Öncel 2002). Relatively to imposition of osmotic stress (Figure 3.18B), the similar effects on germination of wild-type *Ler* and *hrr* mutant suggested that HRR is not implicated in responses to osmotic stress. Together with a HS treatment, the difference observed at 5% of mannitol only corresponds to HS responses, once HRR is involved in responses to HS and, thus not involved in responses to osmotic stress. Considering this, and comparing with salt results (Figure 3.18A), it is most likely that HRR could be more implicated in ionic component of salt stress than in osmotic component.

Salt at higher concentration in cellular apoplast induces ionic toxicity and hyperosmolality. The ionic component is the first cause of ion homeostasis disruption, being sensed by SALT OVERLY SENSITIVE (SOS) pathway components in response to stress. This pathway emphasizes the significance of Ca^{2+} signal in reinstanting the cellular ion homeostasis, by exclusion of excess of Na^{+} ions (Mahajan and Tuteja 2005). SOS pathway compromises three elements: SOS3 (Ca^{2+} binding protein), SOS2 (serine/threonine protein kinase) and SOS1 (target of SOS3-SOS2 complex, which codes for a plasma membrane Na^{+}/H^{+} antiporter-like protein). The perception of salt stress by a Ca^{2+} sensor in plasma membrane elicits cytoplasmic Ca^{2+} pertubations. These are perceived by SOS3, which complexes with SOS2. In downstream pathway steps, SOS2 phosphorylates and activates SOS1. The excess Na^{+} ions are expelled out of the cell and cellular ion homeostasis is restored. In this case, as response to ion deregulation corresponds to early response signalised by Ca^{2+} influx, HRR could be activated at same time, to regulate or protect specific sets of transcripts, such occurs during HS treatment (Figure 3.16).

Germination assays of wild-type *Ler* and *hrr* mutant seeds during oxidative stress conditions could evaluate the role of HRR in leading with high levels of ROS. In this assay, the photosynthetic inhibitor paraquat was included in the MS medium used for seed sowing. This compound interferes with the photosysthem I functioning, generating a high-energy ROS, such as superoxide radical ($O^{2-\cdot}$), and then is converted to hydrogen peroxide (H_2O_2). No significant differences were observed between wild-type *Ler* and *hrr* mutant (results not shown). Hence, the results suggest that HRR is not implicated in responses to oxidative stress, in both experimental conditions.

3.2.5 Hormonal germination sensitivity of *hrr* mutant seeds

The phytohormone ABA plays important physiological and molecular roles in plant growth and development (embryo and seed development, seed desiccation tolerance, dormancy, seed germination, reproduction), as well as in responses to abiotic (drought, cold and salinity) and biotic stresses (Finkelstein *et al.* 2002; Cutler *et al.* 2010). New insights for ABA signalling mechanisms that allow the regulation of many genes have been recently described. The gene expression induction of direct and indirectly ABA-regulated genes contributes for the responses under development signals and environmental cues (Cutler *et al.* 2010). Particularly, ABA acts antagonistically with gibberellins (GA) during seed germination process, where GA positively regulates the germination and ABA inhibits it (Razem *et al.* 2006). The tight and coordinated balance between molecular and physiological levels of both phytohormones is crucial for development transition that happens from stratified to germinated seed.

As discussed in section 3.1, *HRR* is predicted to be expressed during seed development process, (namely during early embryogenesis and then in later stages of seed maturation) and during the endosperm development (Figure 3.9). *HRR* was also suggested to be implicated in thermotolerance responses of HS-stressed seeds during germination (Figure 3.17). For assessing the ABA and GA susceptibility of *hrr* and HRR over-expression mutants, a germination assay was performed in the presence of different exogenous ABA and GA concentrations. These assays will allow to understand the HRR involvement in ABA and GA signalling transduction pathways, during seed development and germination.

3.2.5.1 Germination assays in the presence of ABA

Stratified wild-type *Ler* and *hrr* mutant seeds (4ºC, 2 days) were directly sown onto MS agar medium supplemented with different concentrations of ABA. Germination, evaluated by radicle emergence, was followed during 10 days (Figure 3.19A-D). The germination of *hrr* mutant seeds was always more pronounced than wild-type *Ler* seeds. However, the germination rate differences were more evident between the fourth and sixth day of seed germination. In the high concentration of ABA (2 µM), wild-type *Ler* germination rate was about half of that verified for *hrr* mutant. After the sixth day, the germination rate differences between *hrr* mutant and wild-type *Ler* were less emphasised, reaching a steady-state. Considering the germination rate values obtained by the fifth day, under increasing ABA concentrations, the germination impairment of wild-type *Ler* seeds was evident when compared to *hrr* mutant (Figure 3.19E). The higher germination rate difference observed between

wild-type *Ler* and *hrr* mutant was observed at fifht day of germination for the highest ABA concentrations (1.5 and 2.0 µM).

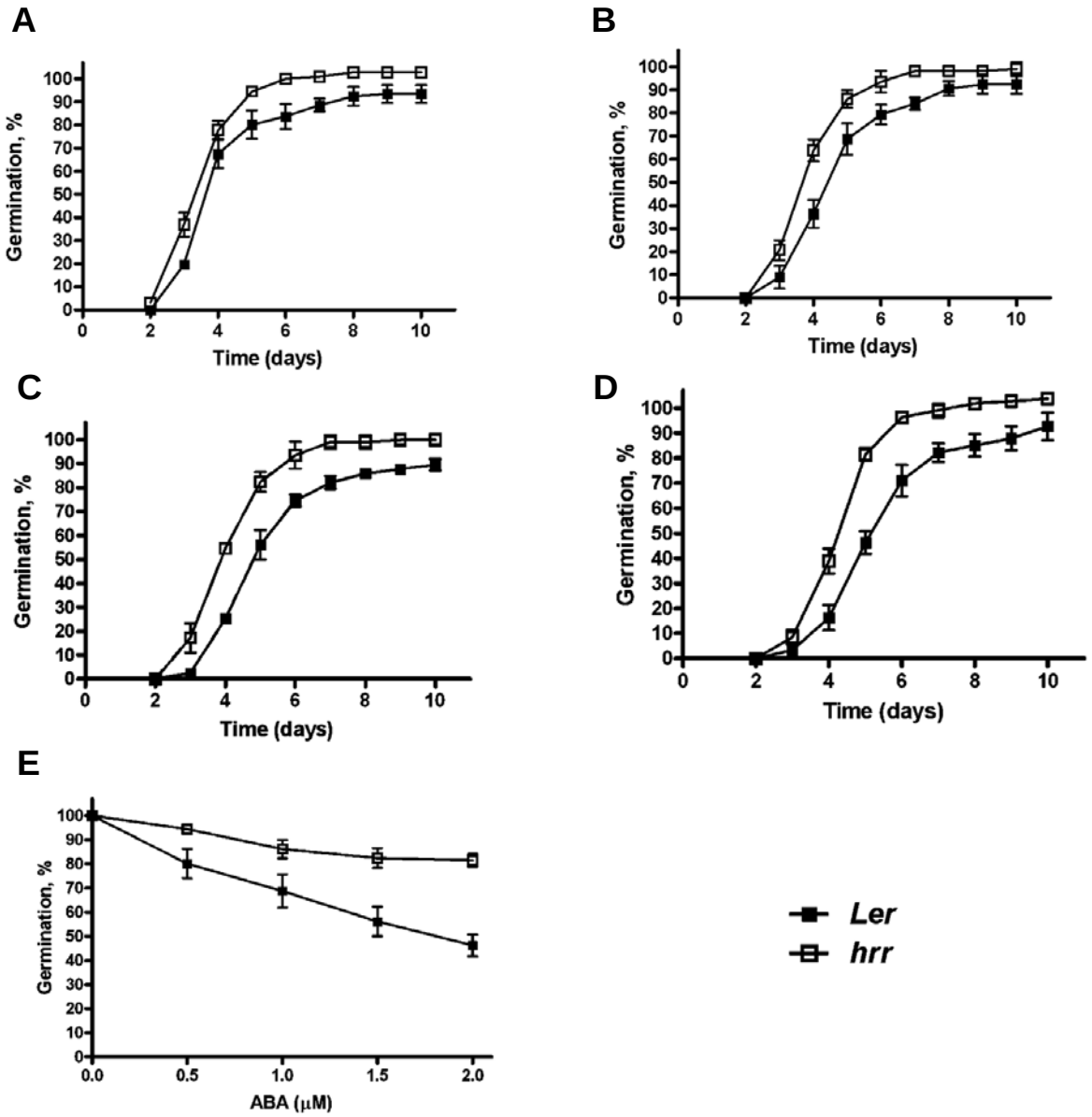


Figure 3.19 Evaluation of ABA effects on *hrr* mutant seed germination. Stratified *hrr* mutant and wild-type *Ler* seeds (4ºC, 2 days) were directly sown onto MS agar medium supplemented with **(A)** 0.5 µM ABA, **(B)** 1 µM ABA, **(C)** 1.5 µM ABA or **(D)** 2 µM ABA. Seed germination was evaluated by the radicle emergence scoring during ten days. **(E)** Effect of different concentration of ABA on wild-type and *hrr* mutant seeds, on the fifth day upon sowing. The results were obtained from four replicates (30 seeds each) and were normalised with corresponding germinated seeds in control conditions (without ABA). Data correspond to means ± SEM and were evaluated through *t*-test analysis, under a significance level of $P<0.05$.

As ABA is required during the embryogenesis process, namely during seed maturation and dormancy induction, the exogenous application of ABA is known to extend the seed dormancy (Jiang *et al.* 2012). The enhanced germination of *hrr* mutant in relation to wild-type *Ler* seeds, in the

presence of ABA, suggests that HRR is a positive regulator on ABA signalling that inhibits seed germination. The genetic data support the idea that, during seed germination, HRR could be involved in ABA signalling pathways. Meanwhile, the application of higher ABA concentrations would give a better understanding about how much the HRR could be involved in regulation of ABA signalling.

Several mutants display ABA-insensitive phenotypes similar to *hrr* mutant. The *abi4*, *abi5* and, in a less extent, *abi3* mutants were described to be insensitive to ABA during seed germination (Finkelstein 1994; Söderman *et al.* 2000; Brocard *et al.* 2002). The activity of these transcription factors is ABA-dependent, acting as principal regulators in the maturation phase of embryo development, thought they are also expressed in some vegetative tissues (Söderman *et al.* 2000). Also, the *ahk1* mutant demonstrated a high germination rate in the presence of ABA, indicating that *ahk1* mutants are also ABA insensitive. The AHK1/ATHK1 gene that codes for a histidine kinase appears to act as a positive regulator of ABA signal transduction, being involved in the phosphorylation of many components of ABA signalling pathway (Tran *et al.* 2007). Indeed, the AHK1/ATHK1 kinase has been proposed to be a ABA signalling component that can sense and transduce a signal of external osmolarity to downstream genes (Tran *et al.* 2007). More recently, the triple mutant *srk2d/e/I* was described to completely block the ABA signalling in germination and post-germination stages, presenting a complete growth development insensitive to ABA (Nakashima *et al.* 2009). These SNF1-related protein kinases has been proposed as being the central positive regulators in ABA signalling during germination, being essential for the control of seed development and dormancy (Nakashima *et al.* 2009).

The role of HRR as a possible positive regulator on ABA signalling, in extending of seed maturation and dormancy mechanisms is corroborated by the results obtained with HRR over-expression lines (Figure 3.20). When seeds of HRR over-expression lines were directly germinated onto MS-agar medium supplemented with exogenous ABA (2 μM), the sensibility responses were different from those obtained with *hrr* mutant. By the fourth day of germination, all HRR over-expression line seeds germinate better than *hrr* mutant and wild-type *Ler* seeds, but in the following days the germination rates become more reduced than in *hrr* mutant (Figures 3.20A-B). By the tenth day of germination, while HRR over-expression lines in *Ler* background (L2 and L6) show a similar germination rate compared to wild-type *Ler*, HRR over-expression lines in *hrr* background (JP5, JP6 and JP9) present a reduced germination rate when compared to wild-type *Ler*. Indeed, L2 and L6 over-expression lines appeared to show similar levels of ABA sensitivity than *hrr* mutant seeds (Figure 3.20B). This result reinforces the premise that these HRR over-expression L2 and L6 lines are silenced by transgenic *HRR* siRNA molecules and develop a phenotype similar to *hrr* mutant (section 3.1).

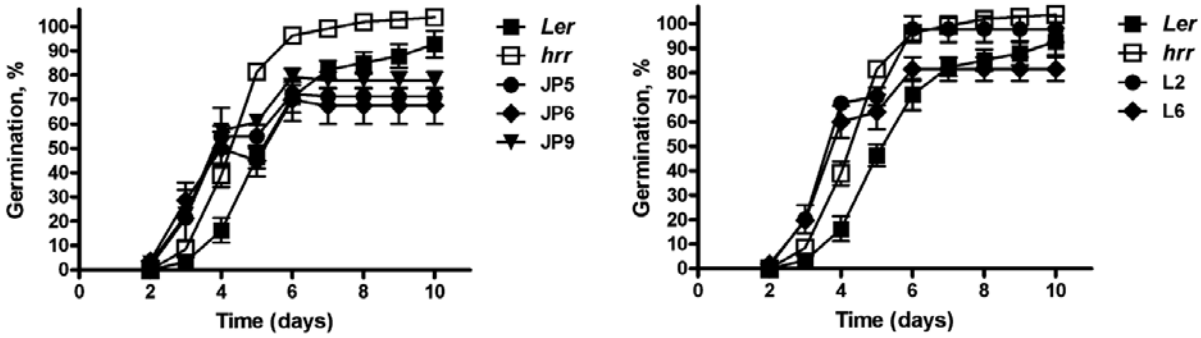


Figure 3.20 Evaluation of ABA effects on (A) JP5, JP6 and JP9 and (B) L2 and L6 HRR over-expression lines. Seeds of wild-type *Ler*, *hrr* mutant, HRR over-expression line in hrr background (JP5, JP6, JP9) and in Ler background (L2, L6) were stratified (4ºC, 2 days) and directly sown onto MS medium containing 2 µM ABA. The germinated rates correspond to radicle emergence scoring taken during ten days. Germinated seeds were normalised with respective germinated seeds in control conditions and their rates obtained from four replicates, 30 seeds each. Data correspond to means ± SEM and were evaluated through one-way ANOVA (Tukey test), under a significance level of 0.05.

The hypersensitivity to ABA displayed by HRR over-expression lines (in *hrr* background) corroborates the hypothesis of ABA insensitivity promoted by HRR. This result is similar to defective mutant in *abh1,* which shows ABA hypersensitivity and reduced wilting during drought (Hugouvieux *et al.* 2001). *ABH1* (abscisic acid hypersensitive 1) codes for an mRNA cap binding protein (homologous to human CBP80) and was suggested to be a negative regulator during the early ABA signal transduction events, playing key roles in mRNA processing of certain ABA-dependent expressed transcripts. Similarly, The *sad1* (*supersensitive to ABA and drought 1*) mutant proved to be ABA hypersensitive, displaying also germination and root growth impairments under drought conditions (Xiong *et al.* 2001; Kucera *et al.* 2005). In addition, *sad1* mutant is also affected in the expression of some stress-responsive genes, particularly ABA biosynthesis genes, like the AAO3 (Abscisic aldehyde oxidase) (Xiong *et al.* 2001; Kucera *et al.* 2005). As *SAD1* codes for a Sm-like SnRNP protein, which could be involved in mRNA splicing, exporting and degradation, SAD1 could play critical roles in regulation of positive feedback loops during the early steps of ABA signalling in stressful conditions (Xiong *et al.* 2001). Also, the *hly1* (hyponastic leaves) mutant exhibits hypersensitivity to ABA. HYL1, corresponding to dsRNA-binding protein, demonstrated to be ABA-regulated, mediating an inhibitory effect at transcriptional or post-transcriptional levels of ABA-related genes during germination process (Lu and Fedoroff 2000).

Altogether, the results suggest that HRR confers insensitivity to ABA. Not only the *hrr* mutant germinate better than wild-type *Ler* seeds (Figure 3.19), but also the over-expression of *HRR* confers some degree of hypersensitivity to ABA (Figure 3.20A). HRR could be positively involved in ABA

metabolism and signalling, possibly acting as a co-regulator and promoting the positive feedback loop during seed maturation and germination in the presence of ABA.

3.2.5.2 Germination assays in the presence of GA

As ABA levels are strictly regulated by raising levels of GA during the germination process. The possible role of HRR in GA signalling pathways was evaluated. Stratified seeds (4ºC, 2 days) from wild-type *Ler* and *hrr* mutant were directly sown onto MS agar medium containing different concentration of GA. The *hrr* sensibility to the presence of this phytohormone is reversed in relation to ABA treatment. The *hrr* mutant germination is more sensitive to GA hormone than wild-type, though the *hrr* germination rate eventually attains similar values to wild-type *Ler* (Figure 3.21). This effect is more pronounced at GA lower concentrations. Indeed, only at lowest GA concentration (25 µM), occurred a significant difference of germination rates was detected. Interestingly, hereafter, with raising GA concentrations, *hrr* hypersensitivity declines and the mutant reaches similar germination rates to those observed in wild-type *Ler*, at highest concentration tested (100 µM).

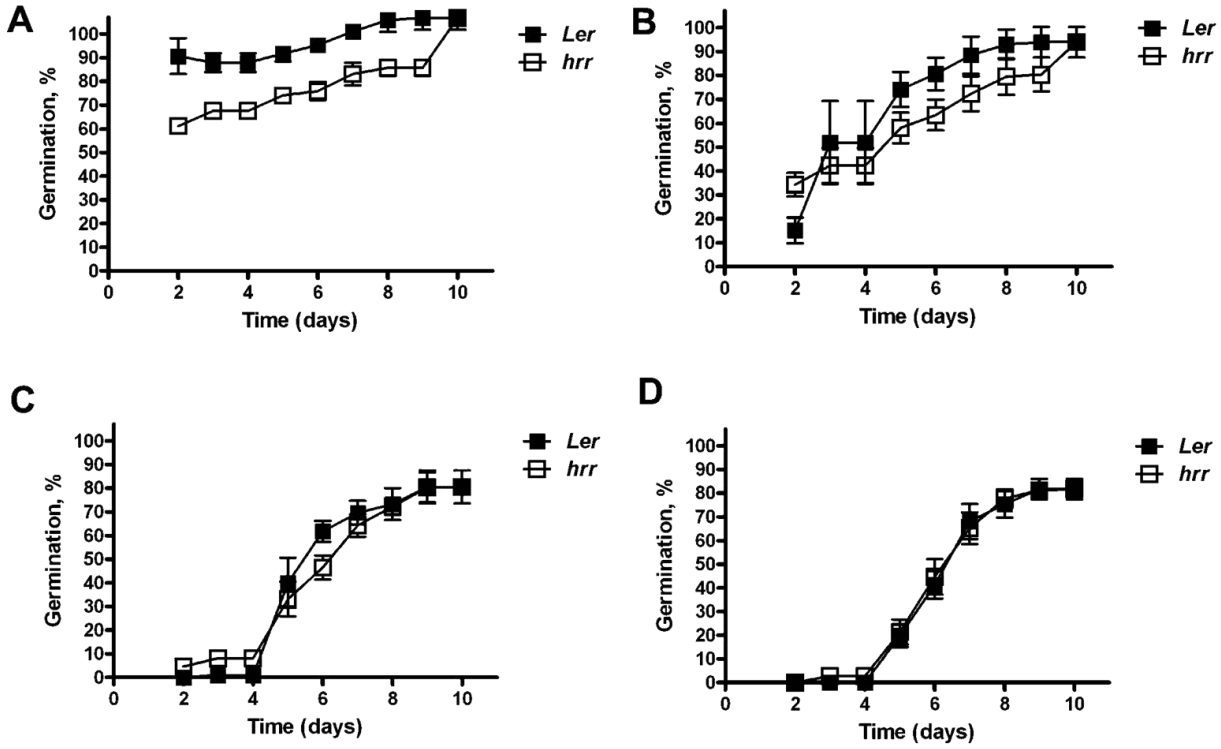


Figure 3.21 Evaluation of GA effects on *hrr* mutant seed germination. Stratified wild-type *Ler* and *hrr* mutant seeds (4ºC, 2 days) were directly sown onto MS agar medium supplemented with **(A)** 25 µM GA, **(B)** 50 µM GA, **(C)** 75 µM GA, **(D)** 100 µM GA. Seed germination was evaluated by the radicle emergence scoring during ten days. The results were obtained from four replicates (30 seeds each) and were normalised with corresponding germinated seeds in control conditions (without GA). Data correspond to means ± SEM and were evaluated through *t*-test analysis, under a significance level of $P<0.05$.

The imposition of increasing GA concentrations mimics the raising levels of GA during seed germination process. The application of exogenous GA lowers the endogenous ABA content by enhancing the ABA catabolism (Okamoto *et al.* 2006). In response to lowest levels of ABA, it is possible a decrease in HRR protein accumulation. Considering this, the application of high exogenous GA concentrations attenuates the GA hypersensitivity observed for *hrr* mutant seeds, which is similar to wild-type *Ler*. The lowest supplied exogenous GA concentration (25 μM) could not be enough to induce the ABA catabolism. At this point it would be important to consider the endogenous GA/ABA ratio, instead of considering each absolute phytohormone amount. In low exogenous GA concentration, the GA/ABA ratio could not be enough to promote the *hrr* mutant seed germination. With increasing concentrations of GA, the endogenous GA/ABA ratio leads to an improvement of *hrr* mutant germination rate compared to wild-type *Ler* attaining similar germination levels at the highest GA concentration. Hence, the reduction of *hrr* mutant hypersensibility to increasing concentration of GA suggests that *HRR* could be down-regulated during seed germination process. At the highest concentrations of GA, the germination rates of both lines are slowly impaired, mostly due to a negative feedback mechanism involved in regulation of GA levels (Figure 3.21C and D). Previous work demonstrated that GA biosynthesis genes (*GA3ox* and *GA20ox*) are down-regulated by exogenous GA treatment and, in contrast, GA catabolism genes (*GA2ox1* and *GA2ox2*) are up-regulated (Sun 2008). This gene expression coordenation shows that GA homeostasis is controlled by a negative feedback mechanism. The *de novo* GA biosynthesis could have been compromised under the highest GA conditions, thus delaying the germination of both wild-type *Ler* and *hrr* mutant seeds. Indeed, it is possible corroborate these results with those for ABA germination assays (Figure 3.19). Under low levels of exogenous ABA, the GA/ABA ratio could be sufficient to promote the germination of both lines, not being detected the *hrr* mutant sensibility. Hence, it is perceptible that *hrr* germination ability is similar to wild-type *Ler*, in both hormonal conditions (Figure 3.21D and 3.19A). Again, these results reinforce the ideia that HRR is a positive regulation of ABA signalling.

Taken together, these results suggest that HRR is a RNA-binding protein strongly involved in plant thermotolerance responses, according with predicted bioinformatic data (section 3.1). However, contrary to bioinformatic data, under these experimental conditions, HRR appear to be involved in responses to salt stress imposition, under the ionic impairment condition imposed by high apoplastic salinity. HRR is not involved in responses to osmotic and oxidative stress conditions. Once the transition phase of dormancy for seed germination is strictly regulated by hormonal ratio levels of ABA and GA, HRR seems to function as a positive regulator in ABA signalling pathway, whereas

appears to exert negative effects in GA signalling. Once ABA is considered a phytohormone of stress and HRR is exclusively expressed under stressful conditions (HS), it is agreed the positive role of HRR in ABA signalling.

Being a putative stress-responsive RNA-binding protein, HRR might be important for the post-transcriptional regulation during seed maturation and early desiccation stages, particularly characterised by the water deficit conditions and accumulation of organic compounds (proteins, nucleic acids, lipids, sugars). This progressive water content reduction and concomitant increasing of reserve accumulation leads to low oxygen content (anoxia) in seed. Together, these conditions compromise several post-transcriptional mechanisms. So, the processing, stability, transport and proper storage of mRNAs would be of major importance for the establishment of early steps of seed germination, either under HS conditions or other combined stress treatments. Therefore, HRR could be a relevant RNA-binding protein involved in many of mRNA metabolism steps (pre-mRNA processing, mRNA transport and stability, translation initiation process) that could have place during the developmental transition from seed to seedling.

During the early steps of seed germination, where the temperature and light are also fundamental, *de novo* mRNA synthesis is imposed. RNA-binding proteins, possibly including HRR, would be important for regulation of the levels of these newly transcripts, thus controlling the germination onset. *HRR* could be up-regulated in the early phase of imbibition, where a transient rise in ABA content occurs in the embryo (Sun 2008). This could be sufficient for the induction and regulation of transcript levels of ABA signalling factors, such as ABI factors, which play crucial roles in transition phases during germination. As ABA content decrease, the embryo growth is promoted by *de novo* GA biosynthesis, due to the up-regulation of GA biosynthesis and signalling genes.

3.2.6 Complementary data

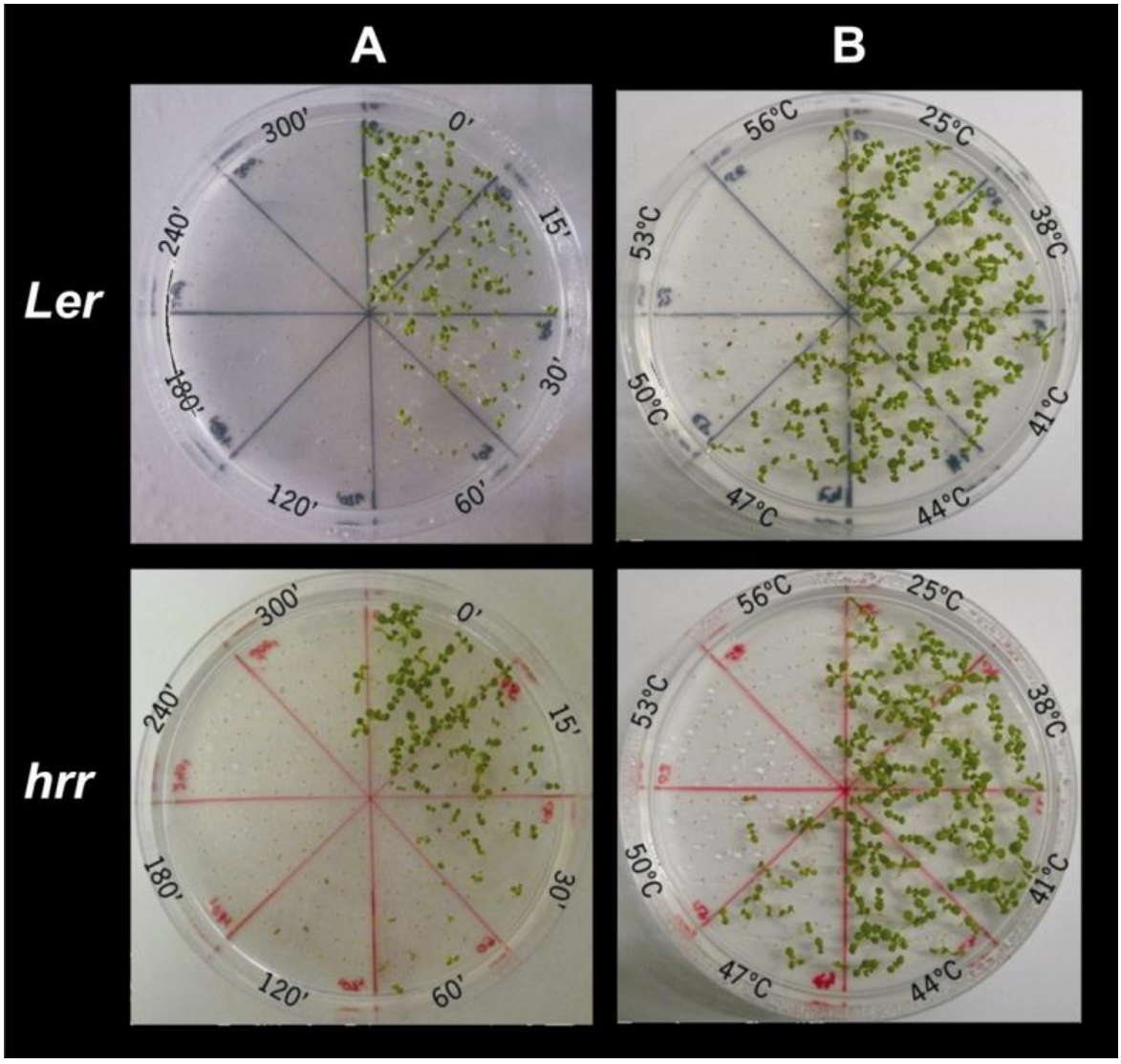


Figure C3.5 Heat sensibility of wild-type *Ler* and *hrr* mutant after HS treatments. After stratification (4ºC, 2 days) and before sowing onto MS medium, wild-type *Ler* and *hrr* mutant seeds were **(A)** heat-stressed at 50ºC, for periods ranging from 0 (control) to 300 min and **(B)** heat-stressed for 60 min, at different temperatures, ranging from 23ºC (control) to 56ºC. Photographs were taken 10 days after heat treatment.

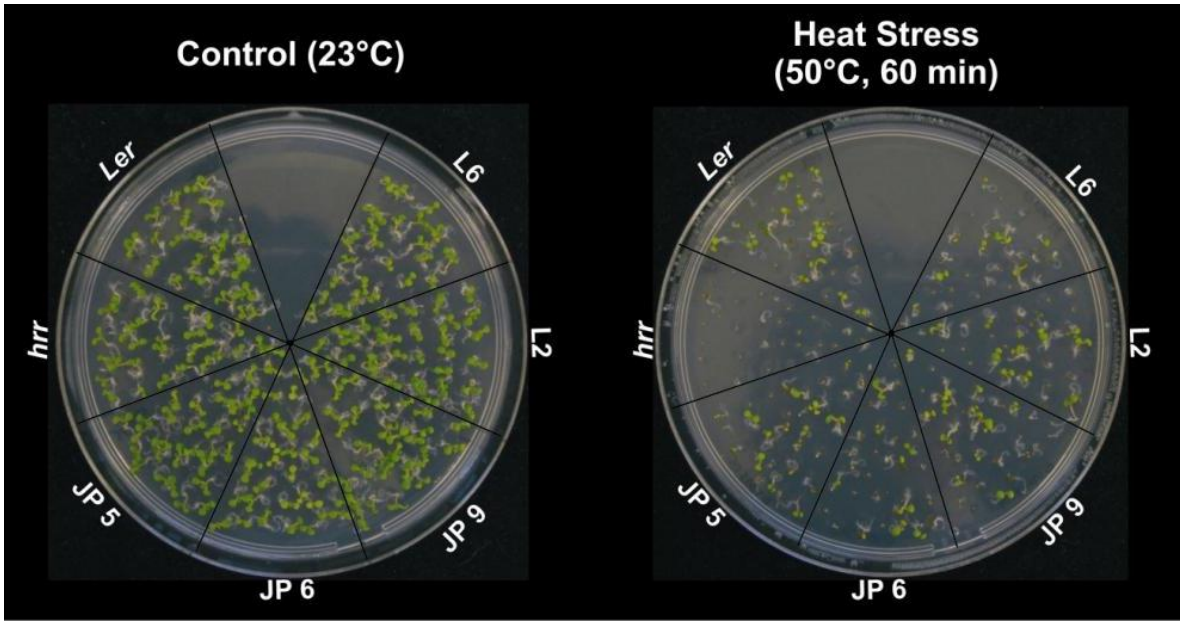


Figure C3.6 Complementation of *hrr* mutation and effect of over-expression of *HRR.* After stratification (4ºC, 2 days), seeds from wild-type *Ler*, *hrr* mutant and different *p35S::HRR-GFP* transgenic lines were heat-stressed (50ºC, 60 min) and directly sown onto MS medium. JP5, JP6 and JP9 refer to transgenic lines in *hrr* background. L2 and L6 refer to transgenic lines in *Ler* background. Photographs were taken 10 days after heat treatment.

3.3 *HRR* gene expression and their putative roles in regulation of HS- and plant developmental-related transcriptomes

The analysis of accessed ATH1 microarray data revealed that *HRR* is strongly induced just after HS imposition (*BAR- The Bio-Array Resource for Plant Biology*, http://142.150.214.117/welcome.htm). The maximal expression levels were detected in roots, one hour after HS treatment, being reduced to basal levels during the recovery period (Section 3.1). In attempting to confirm these *in silico* data, *HRR* expression analysis was performed, both in seedlings (16-days-old) and imbibed seeds. In addition to its higher expression under HS conditions, the bioinformatic data predicted that *HRR* is also expressed during later stages of seed maturation and during seed germination (Section 3.1.3). Considering these facts, *HRR* expression profile was evaluated during different stages of seed development and in seed germination. In perspective of putative HRR functions under HS conditions and those plant development stages, becomes important to understand how much HRR could be involved in regulation of expression levels corresponding to specific set of genes. Hence, the *hrr* mutant and HRR over-expression lines were used to determine respective expression profiles. In response to a variety of stresses, many plant transcripts undergo to alternative splicing mechanisms. Based in results obtained for *HRR* expression analysis, a mRNA decay analysis of HRR alternative transcripts was performed. The HRR funcional prediction was complemented with histochemical analysis, to access in which organs/tissues the *HRR* expression occurs, through the *HRR* promoter activity.

Collectively, the presented results focus for attribution of putative HRR functions in regulation of the HS-induced transcriptome, as well as in the seed development and germination transcriptomes.

3.3.1 Heat-dependent *HRR* expression analysis, in seedlings

To verify if *HRR* expression is dependent of HS, a semi-quantitative RT-PCR was performed to follow the accumulation of *HRR* transcripts during HS treatment and subsequent recovery, at 38ºC, in 16-days-old wild-type (*Ler*) seedlings (Figure 3.22A). The *HRR* transcript levels were induced from one to three hours upon HS treatment and returned to basal levels after three hours at recovery temperature (23ºC). Two alternative-spliced *HRR* transcripts were observed, which will be hereafter designated as *HRR.1* (510 bp) and *HRR.2* (583 bp). Both transcripts were detected just after 30 min of HS and reached the highest levels after 60 min of HS imposition, where the longest transcript

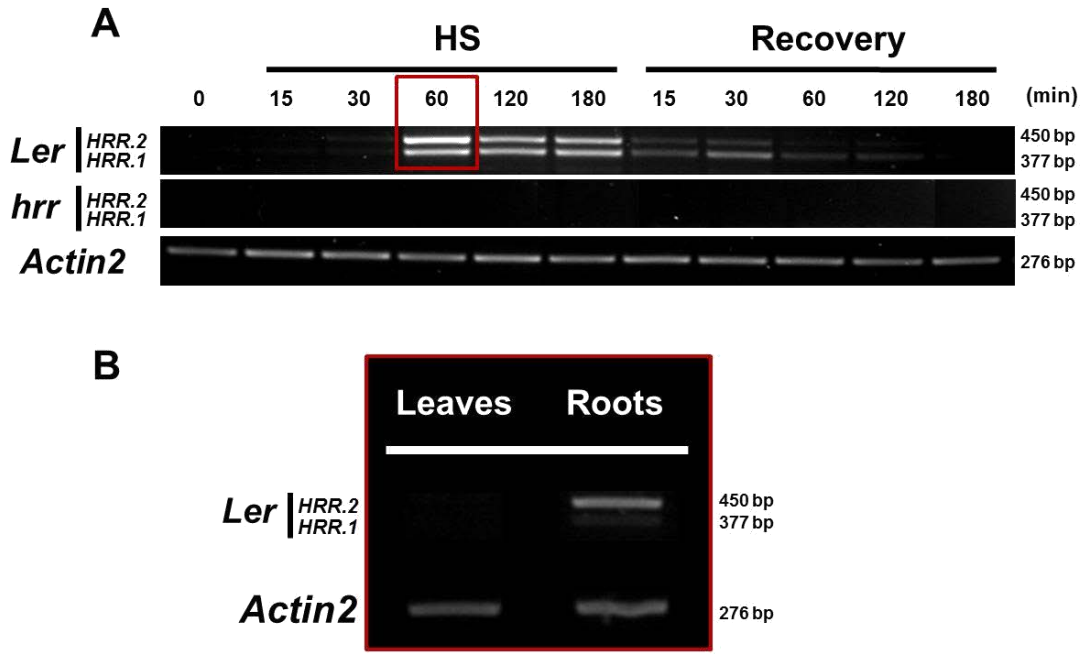


Figure 3.22 Expression analysis of *HRR* during heat stress imposition and recovery. (**A**) Transcript levels were determined by semi-quantitative RT-PCR from mRNA extracted from HS-treated 16-days-old seedlings (wild-type *Ler* and *hrr* mutant). Seedlings were heat-stressed (HS, 38ºC) for periods ranging from 15 to 180 minutes, or heat-stressed for 180 minutes and then allowed to recover (23ºC) for different periods up to 180 minutes. (**B**) Differential expression of *HRR* gene in wild-type *Ler* roots and shoots was determined using mRNA extracted from heat-stressed (38ºC for 60 min) 16-days-old seedlings. As internal controls, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

(*HRR.2*) displayed higher expression than the other (*HRR.1*). In contrast, during recovery, *HRR.2* transcript levels declined more rapidly than *HRR.1* transcripts. Indeed, after 30 min of recovery, only a reduced level of *HRR.2* is visible, while *HRR.1* expression still endures up to 120 min. When performing RT-PCR analysis using *hrr* mutant seedlings in the same HS conditions, no expression of *HRR* gene was detected, confirming *hrr* as a knockout mutant of *HRR* (Figure 3.22A).

As the microarray data analysis indicated that *HRR* expression was maximal in roots (Figure 3.1), in order to examine in which seedling organs the *HRR* expression is highest, a similar expression analysis was performed in leaves and roots of heat-treated (38ºC, 60 min) seedlings. As expected, though the same overall expression pattern of alternative-spliced transcripts was observed, both *HRR* transcripts were mainly expressed in roots, being quite undetectable in seedling leaves (Figure 3.22B).

The results indicate that under HS an alternative splicing (AS) process produces two different alternative-spliced mRNAs: *HRR.1* and *HRR.2*. *HRR.1* has been annotated as the unique *HRR* transcript in general databases (TAIR, Ensembl), being the *HRR.2* described for the first time in this work. The alternative splicing mechanism, an intron retention process, produces a longer transcript (*HRR.2*, 583 bp) that retains the first and smaller intron (Figure 3.23). As this transcript

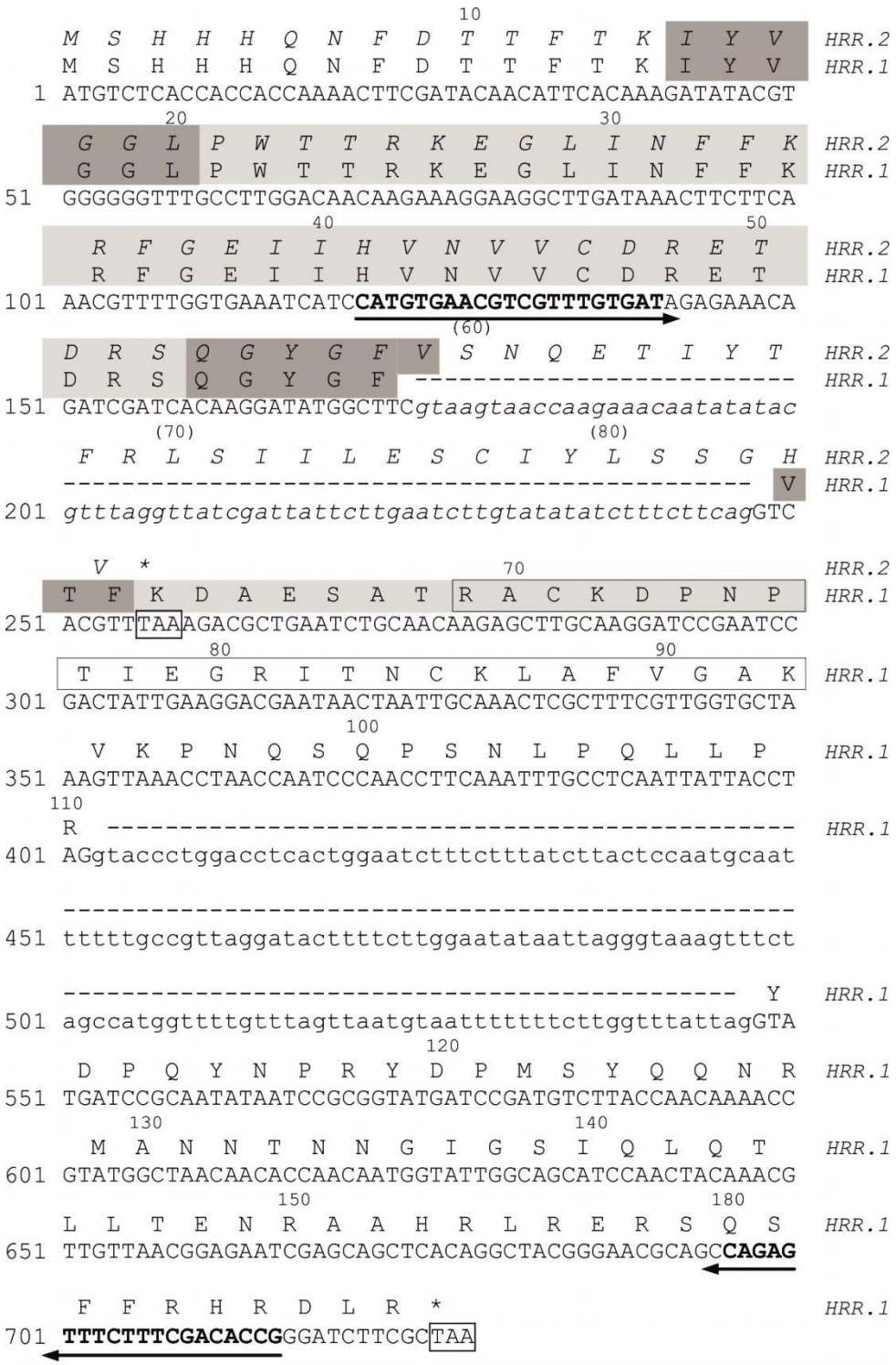


Figure 3.23 *HRR* gene structure and deduced amino acid sequence of HRR proteins. Introns are represented in lower case and the one that is retained in the alternative splicing mechanism, resulting in the *HRR.2* transcript, is displayed in italics. The predicted amino acid sequence of HRR.1 and HRR.2 isoforms are depicted above the nucleotide sequence. Both HRR proteins present a RRM domain (shadowed in light grey with the corresponding RNP consensus sequences, RNP2 and RNP1, in dark grey), but only HRR.1 protein contains the PABP-1234 domain (boxed). All protein domains were predicted by NCBI. Numbers on the left refer to nucleotides, and numbers above amino acid sequences refer to amino acids (in italic for HRR.2). Black arrows and bold letters represent the primers used in RT-PCR for expression analysis.

harbours an in PTC, HRR.2 is predicted to be 84 amino acid residues shorter than HRR.1. Both HRR proteins contain a RRM domain, described as a RNA-recognition motif, which contains the two highly conserved consensus sequences: the hexamer RNP2 and octamer RNP1 (Figure 3.23, shadowed in dark grey). As the alternative splicing site interrupts the coding region of RNP1, a slight difference in the amino acid sequence occurs in both isoforms (QGYGFVSN in HRR.2; QGYGFVTF in HRR.1) (Figure 3.23).

The expression analysis corroborates the microarray data, since *HRR* up-regulation was observed after one to three hours of HS treatment, being mostly detected in roots (Figure 3.22). The differential *HRR* expression in roots and shoots could be explained by the difference in temperature sensitivity and distinct function played by tissues that lead to different protein synthesis pattern (Huang and Xu 2008).

Up to now, the possibility of AS for *HRR* transcripts has never been predicted. The AS mechanism by intron retention, is considered to be the main AS event in plants, comprising about 41% of AS in Arabidopsis, contrasting with human genes, where only 9% follow by an intron retention process (Barbazuk *et al.* 2008). When the AS occurs by an intron retention process, alternatively retained introns can appear as part of coding sequences (CDS) or bridging the CDS and UTRs, or even be located at the 5' or 3' UTR (Ner-Gaon *et al.* 2004; Louzada 2007). Many transcripts containing a retained intron have been related to stress or other stimuli input, exerting many effects on their own stability and nuclear transport or even in other transcripts (Ner-Gaon *et al.* 2004). Thus, the imposition of an environmental cue, such as HS treatment, would promote not only modifications in transcription, but also in splicing processes or protein modifications. The production of *HRR.2* alternative transcript could mainly result from effects in the spliceosome composition and activity under heat stress conditions. Alterations in the spliceosome machinery (composition, concentration, activity) could change the splicing pattern of transcripts. Consequently, the subsquent transcription and post-transcription events could be modified, altering the expression and splicing of downstream expressed genes needed for metabolic and development processes (Simpson *et al.* 2008). The occurrence of such spliceosome modifications, will change the recognition of 5' and 3' splice sites (ss) and the splicing of *HRR* introns, resulting in the production of two different alternative transcripts. The possible occurrence of small changes in intron splicing signals could influence the binding and activity of the spliceossome complex. Plant intron 5' and 3' ss consensus sequences are very similar to those of vertebrate introns, but they exhibit a great variation around the conserved :GU and AG: dinucleotides at 5' and 3' ss, respectively (Brown 1996; Brown and Simpson 1998). Sequencing results for *HRR.2* sequence revealed that those consensus sequences in alternative splice sites are

not altered, occurring only variations in neighbour nucleotides. Perhaps these small variations would determine the recognition and activity strengths of spliceosome complex.

The production of *HRR.2* alternative transcript, under HS conditions, could exert important effects in transcript stability and translation initiation processes. If the corresponding mRNP complex passes through the checkpoint at nuclear transport level (described as 'pionner round'), in the cytoplasmic side of nuclear membrane, the presence of an in-frame PTC in *HRR.2* could be a further signal for translation blocking. Such mechanism could influence the transcription of *HRR* gene, probably being responsible for the own down-regulation. Indeed, the presence of misspliced introns in certain transcripts functions as a signal for their own down-regulation. Previous works have demonstrated that intron-retained transcripts are mostly associated with polyribosomes, indicating that these transcripts might play regulatory functions in RNA metabolism (Ner-Gaon *et al.* 2004). In mammalian, if an exon junction complex (EJC, which is depositated 20-25 nt upstream of each exon-exon junction) is present more than 50-55 nt downstream from a PTC, the molecular mechanism of NMD can come into play (Ner-Gaon *et al.* 2004; Isken and Maquat 2007). Analysing the alignment of both *HRR* sequences, the PTC is about 150 nts upstream of last exon-exon junction (Figure 3.23), indicating that *HRR.2* alternative transcript could be a potential target for NMD. Further experiences to analyse the *HRR.2* mRNA decay are important to verify if *HRR.2* transcript is removed by NMD-associated mechanisms.

3.3.2 Heat-dependent *HRR* expression analysis, during seed imbibition

Once described the thermotolerance phenotype for HRR, through the basal termotolerance germination assays (Figure 3.16), expression analyses by semi-quantitative RT-PCR were performed in imbibed seeds of wild-type *Ler*, *hrr* mutant and the HRR over-expression independent lines JP9 and L2. After the stratification period, imbibed seeds (control conditions, 23ºC) of each line were then heat-stressed at 50ºC, during one hour. Under control conditions, *HRR* displayed expression in wild-type *Ler*, but none transcript was detected in *hrr* mutant seeds. When submitted to HS treatment, *HRR* was up-regulated in wild-type *Ler* and, as previously demonstrated (Figure 3.22), the HS-treated *hrr* mutant seeds did not express *HRR* transcripts (Figure 3.24). Under these HS conditions, *HRR.2* transcripts were not expressed. In control conditions, JP9 seeds expressed higher *HRR* transcripts than L2 wild-type *Ler* seeds (Figure 3.24). When the HRR over-expression seed lines were subjected to HS treatment, the *HRR* transcripts were differently expressed. In case of JP9 seeds, it was observed that occurred a slight reduction of *HRR.1* transcript levels, whereas in L2

seeds displayed an up-regulation of *HRR.1* transcripts. Indeed, both HS-treated JP9 and L2 seeds expressed similar *HRR.1* transcript levels, when compared with wild-type *Ler*.

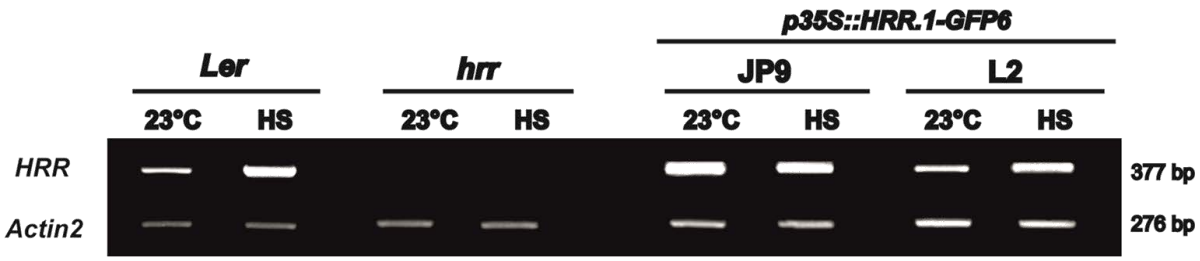


Figure 3.24 Expression analysis of *HRR* during heat stress imposition, in imbibed seeds, subjected or not to HS. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from imbibed wild-type *Ler*, *hrr* and HRR over-expression (JP9 and L2) mutant seeds, which were subjected to HS treatment (50ºC for 60 min) or were maintained at standard conditions (23ºC). As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair primers and PCR conditions are depicted in Annexes III and IV, respectively.

Under control conditions, the JP9 lines showed the highest *HRR.1* transcript levels, in comparison with other lines. Meanwhile, *HRR.1* transcript levels in L2 seeds were similar to those in wild-type *Ler*. These results indicate that ectopic expression of *HRR.1* is greatly influenced by seed genetic background. Thus, as in imbibed wild-type seeds occur *HRR* expression, the introduction and over-expression of *HRR* transgene could lead to induction of silencing mechanisms. As *hrr* mutant is a knockout line, the *HRR.1* over-expression in this line (JP9 seeds) leads to accumulation of *HRR* transcripts.

In this expression analysis, *HRR.2* transcripts were not expressed in HS-treated wild-type *Ler* seeds, such as occurred in wild-type *Ler* seedlings (Figure 3.22). This result could be explained by differences of developmental stage and experimental conditions used. Under HS conditions, *HRR.1* transcripts analysed in JP9 seeds appeared to be down-regulated, relatively to control conditons. Indeed, in same conditions, *HRR* transcript levels are similar in wild-type *Ler* and HRR over-expression JP9 seeds. The reduction of *HRR* transcript levels in JP9 seeds could be due to accumulation of cytoplasmic mRNP aggregates, as result of activation of defense mechanisms against stressful conditions.

3.3.3 *HRR* expression during seed development and germination

The *in silico* gene expression analysis predicted the *HRR* up-regulation during seed development and germination processes (Section 3.1.3). Moreover, the basal thermotolerance phenotype observed during seed germination (Section 3.2.3) could result from deregulation of seed

development process in *hrr* mutant. The seed germination process is characterised by the transition of seed from dormant to non-dormant state, under optimal environmental conditions (light, temperature, nutrients). Although the term 'germination' has a surprisingly large number of meanings, the strict sense (*sensu stricto*) meaning corresponds to the period from the imbibition of dry seeds until the embryo (usually the radicle) first emerges from any tissues enclosing it (Nonogaki *et al.* 2007). In this work, germination was considered as the process occurring just after sowing in MS medium until radicle emergence (2d).

To evaluate the expression of *HRR* during seed maturation and subsequent germination, siliques and germinated seeds were harvested during different development stages. The Arabidopsis organ harvesting was carried out considering the correlation between embryo development phases and siliques growth (Figure 3.25). The early stages of embryogenesis occur in silique stages 1 and 2; stages 3 through 5 match the seed maturation stages; and the late maturation of embryogenesis corresponds to pre-desiccated siliques.

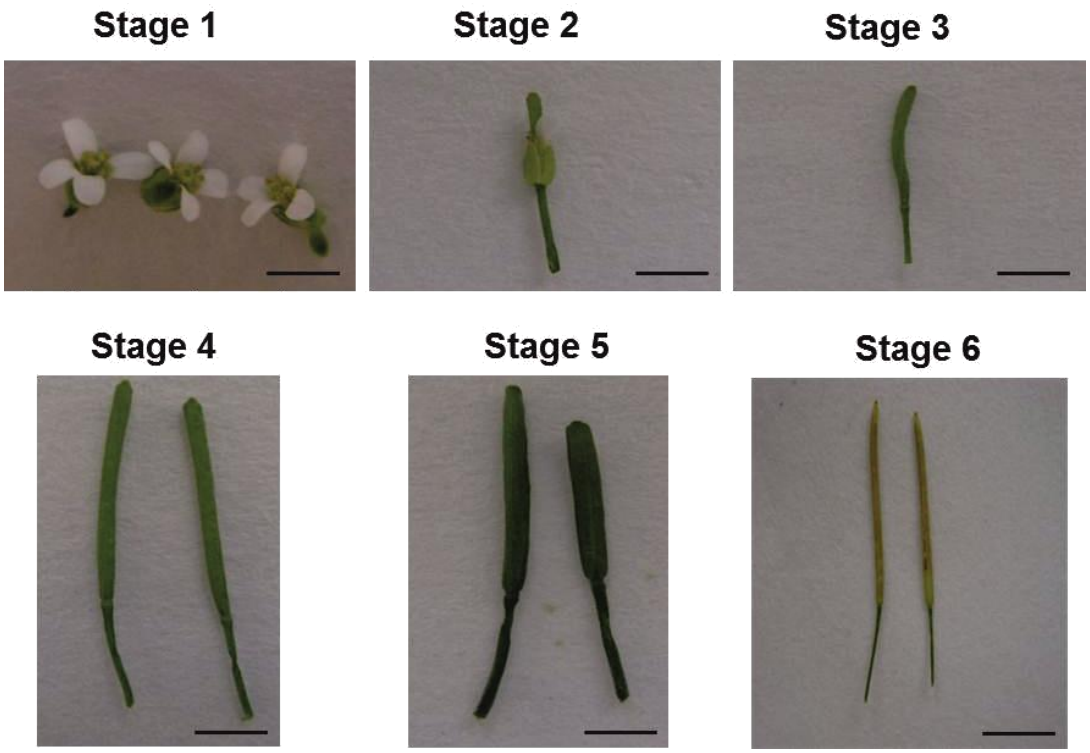


Figure 3.25 Seed development and maturation stages used for semi-quantitative RT-PCR analysis. These stages were chosen according with Arabidopsis eFP browser data (development map, BAR) for *HRR* expression. (1) opened flower, (2) emerged siliques, (3) early siliques, (4-5) developing siliques, (6) mature silique (desiccated silique). Scale: 1 cm.

In the wild-type *Ler*, *HRR* transcript levels were only detected in later stage of seed maturation, corresponding to early phase of desiccation tolerance acquisition (Figure 3.26A).

Twenty-four hours after sowing of imbibed seeds, weak *HRR* expression levels were observed, not being detected any transcript levels at the second day of germination (Figure 3.26B). As expected, any *HRR* expression was not found for *hrr* mutant siliques/seeds.

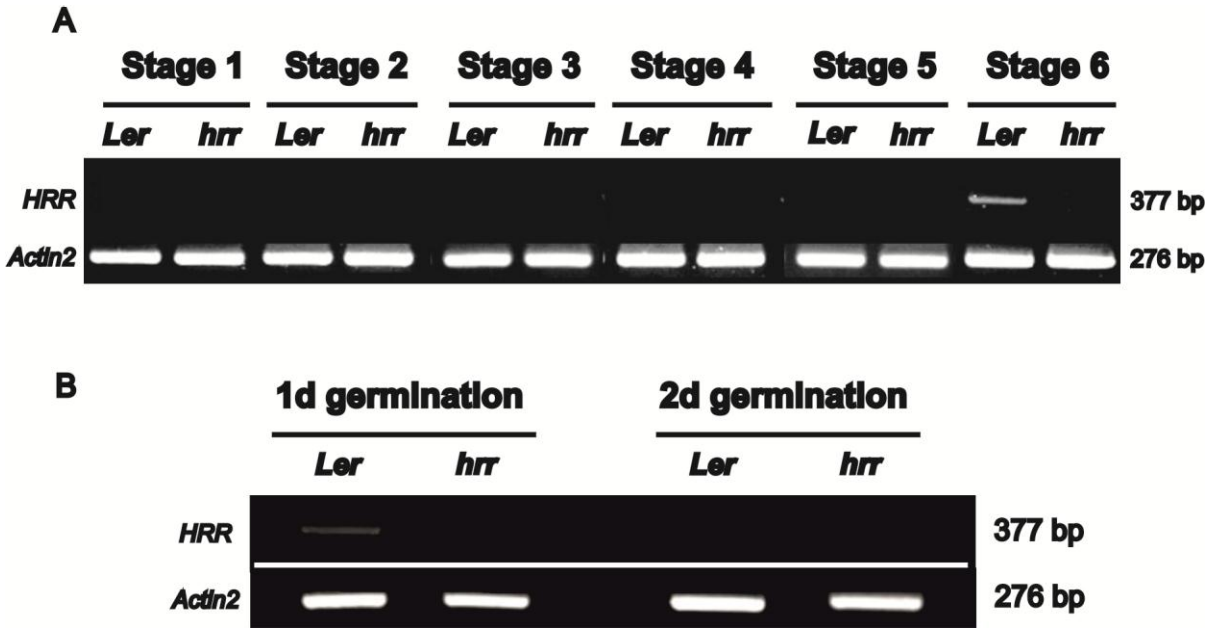


Figure 3.26 Expression analysis of *HRR* during seed development and germination. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted (**A**) from flower/silique tissues, according to defined seed development and maturation stages (Figure 3.25) or (**B**) from germinated seeds with one or two days upon sowing in MS-agar medium. For comparison experiments were performed in wild-type *Ler* and *hrr* mutant. As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

Although *HRR* expression was predicted during early stages of embryogenesis (embryo globular phase), the samples corresponding to stages 1 and 2 might not have included this phase and thus detectable *HRR* expression levels could not have been observed. However, hypothesis of dilution of *HRR* expression signal should not be discarded, due to the specific expression of *HRR* in embryo tissues (globular embryo and peripheral endosperm tissues, Figure 3.9B). Since *HRR* is only expressed in later stages of seed maturation, these results corroborate the *in silico* data (Figure 3.9A).

During germination process, *HRR* expression levels were only detected at first day of germination, almost at basal levels. This result indicates that increasing levels of GA, normally verified during germination, could lead to *HRR* down-regulation. Thus, this result corroborates with negative effect of HRR in GA signalling, during germination process (Section 3.2.5.2). Simultaneously, the increasing of ABA catabolism could be a physiologic order for reduction of HRR activity, once it has been indicated as a positive regulator of ABA metabolism and signalling (Section 3.2.5.1).

Globally, the results demonstrate that *HRR* is highly expressed during HS treatment, thus corroborating with previous *in silico* data (Section 3.1). However, *HRR* expression profile under these stressful conditions depends on plant development stage. In seedlings, the *HRR* expression under HS resulted in production of two alternatively-spliced transcripts: the canonical *HRR.1* and the *HRR.2* alternative transcript (Figure 3.22). The *HRR.2* transcript harbours the first intron of gene, resulting of the intron retention mechanisms. On the other side, in imbibed seeds, only the *HRR.1* transcript was expressed.

During seed development and germination, the *HRR* expression was detected in later stages of seed maturation (Figure 3.26A), imbibed seeds (Figure 3.24) and germinated seeds (first day, Figure 3.26B). These results suggest that HRR could be recruited during early stages of desiccation process, being possibly involved in mRNA storage. The stability of stored mRNAs during the seed desiccation process is fundamental, once the integrity and correct folding of mRNA molecules should be tightly regulated for a proper induction of further seed germination process. HRR could be similarly important for stability of transcripts during transition phase, from dormant seed to germination.

3.3.4 Expression analysis of specific genes in *hrr* mutant and *HRR* over-expression lines

The transition phases in different development stages and responses to the multiplicity of stresses imply the up- and down-regulation of specific sets of genes. The *HRR* up-regulation during HS treatment and during seed maturation and germination leads to investigate if HRR is involved in regulation of specific sets of transcripts. The transcript levels analysed correspond to HS-induced genes, seed-specific TFs, stress-related proteins and ABA/GA metabolism components. The results obtained could further predict HRR functions in consecutive changes of transcriptomes, both under HS and during seed development and germination.

3.3.4.1 HS-related genes

The HS responses are mainly built by the expression of multiple transcription factors (HSFs) and, in turn, HSPs. Together, these HS-responsive components play key roles in plant thermotolerance. Once *HRR* is up-regulated under HS conditions, becomes crucial to understand if HRR could be involved in regulation of the *HSF* and *HSP* transcripts. To study the relevance of predicted HRR proteins in gene expression regulation of *HSFs* and *HSPs*, the transcript levels of

HSFA2, *HSP101, HSP18.1, HSA32* and *HSP25.3* were evaluated by semi-quantitative RT-PCR during heat treatment and following recovery (Figure 3.27). For comparison, this analysis was performed using wild-type *Ler* and *hrr* seedlings (16-days-old). In both lines, all the assayed genes transcripts were only expressed upon HS treatment, though *HSA32* presented slight expression levels in control conditions. The highest *HSP18.1* and *HSA32* transcript levels were achieved during the recovery period. When compared to wild-type *Ler*, the *hrr* mutant exhibited reduced levels of *HSFA2, HSP101* and *HSP18.1* transcripts. This result was more evident during recovery period for the first two genes. In contrast to all other *HSP* genes, *HSP25.3* and *HSP32* transcript levels were increased in *hrr* mutant, not only during the HS imposition but also during the recovery period.

HSFA2 has been described as a heat-inducible *trans*-activator that promotes the maintenance of *HSP* gene expression and extends the duration of acquired thermotolerance in Arabidopsis (Schramm *et al.* 2006; Charng *et al.* 2007). During the fast induction of *HSFA2* gene, the resulting transcripts must be maintained in a stable state, due to their importance for the induction of other downstream HS-responsive genes, thus promoting the thermotolerance extension. During recovery period, the lower *HSFA2* transcript levels in *hrr* mutant, comparing to wild-type *Ler*, could explain the low induction of *HSP* genes, namely *HSP101* and *HSP18.1*, which have been suggested as strong targets of HSFA2 activity under HS treatment (Schramm *et al.* 2006; Charng *et al.* 2007). The *hsfA2* mutant transcriptome profile performed in heat-stressed seedlings (44ºC, 45 min) revealed that *HSP18.1(-CI)* and *HSP25.3(-P)* transcript levels (in this work referred respectively as *HSP18.1* and *HSP25.3*) were the most negatively affected (Charng *et al.* 2007). Indeed, *HSP101* and *HSP18.1* transcripts were down-regulated in *hrr* mutant, during HS imposition and during recovery period.

HSP101 is largely known as molecular chaperone belonging to the AAA+ ATPases class family, involved in development of thermotolerance in plants. The expression pattern of *HSP101* is similar to LEA proteins and *sHSP* genes, during the late seed maturation and/or early germination (Xiong *et al.* 2001). Moreover, HSP101 protein seems to be crucial in basal thermotolerance during germination, since it was predicted to assist the resolubilisation of protein aggregates during HS treatment (Queitsch *et al.* 2000). In *Pisum sativum*, the cytoplasmic and small HSP18.1 was indicated as being involved in the refold of damaged proteins, in co-operation with other HSPs and sHSPs. Low levels of these proteins during early recovery period could be insufficient to promote the refolding of damaged proteins and could target the proteins irreversibly damaged to the proteosome-dependent pathway. Thereby, the importance of HRR function under HS treatment, and mainly in recovery time, might be essential for regulation of key thermotolerance-related transcripts, as are *HSFA2* and, subsequently, *HSP101* and *HSP18.1*.

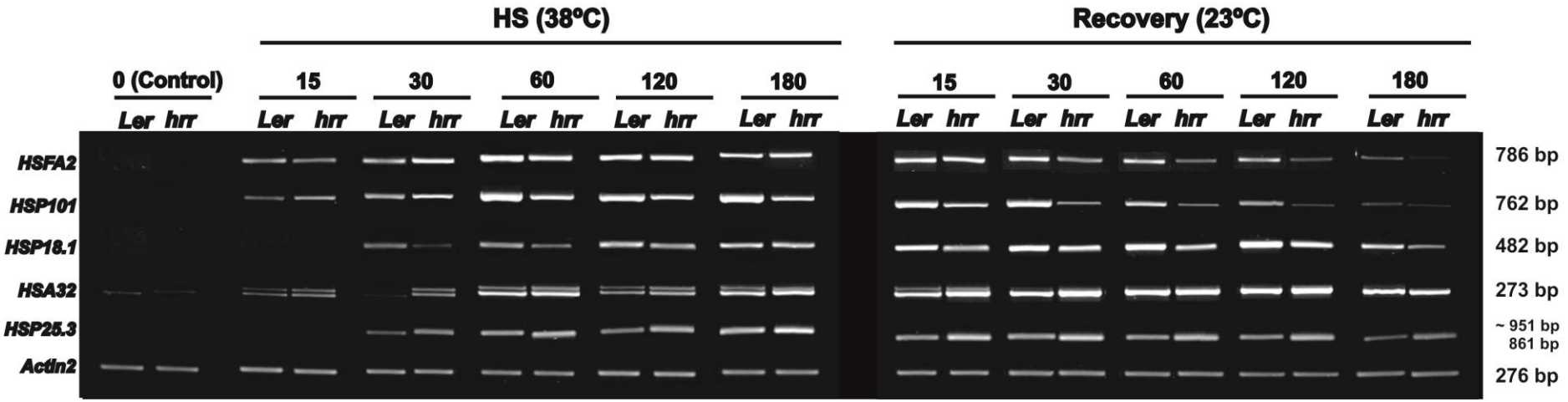


Figure 3.27 Expression analysis of HS-specific genes during heat stress imposition and recovery. Transcripts levels were determined by semi-quantitative RT-PCR from mRNA extracted from heat-treated 16 day-old seedlings (wild-type *Ler* and *hrr* mutant). Seedlings were heat-stressed (HS, 38ºC) for periods ranging from 15 to 180 minutes, or heat stressed for 180 minutes and then allowed to recover (23ºC). Seedlings grown at control conditions (23ºC) were used for comparison. As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

The *HSA32* expression analysis suggests that this protein is substantially expressed during recovery periods. HSA32 has been described as a novel and plant-specific HSP that is involved in improvement of acquired thermotolerance (Charng *et al.* 2006; Liu *et al.* 2006a; Liu *et al.* 2006b). The existence of *HSA32* alternative-spliced transcripts, during HS treatment (15 to 180 min) and early recovery period (15 min of recovery) was already reported but one of the alternative transcripts soon disappeared (30 min of recovery) (Charng *et al.* 2006; Liu *et al.* 2006b). In this work, the *HSA32* transcript levels did not present significant differences, among wild-type *Ler* and *hrr* mutant. Although a reduction of *HSA32* transcript levels has been described in *hsfaA2.1* mutant after four hours of recovery (Charng *et al.* 2007), the low levels of *HSFA2* in *hrr* mutant does not seem to affect *HSA32* expression levels in HS treatment and recovery. Indeed, the slight up-regulation of *HSA32* during recovery time could be due to transactivation by other HSFs rather than HSFA2. Recently, *HSA32* up-regulation under HS conditions was described to be HSFA1-dependent (Liu *et al*.2011). As HSFA1s have been considered as early HSFs, it is likely that *HSA32* could be directly or indirectly activated by these HSFs, under these experimental conditions.

HSP25.3 has been described as a direct HS-responsive gene, whose expression is HSFA2-dependent and could be regulated by HSFA1s. Such as occurs for *HSA32*, *HSP25.3* gene could be also activated by HSFA1s, when *HSFA2* expression is impaired (Schramm *et al.* 2006; Charng *et al.* 2007; Liu *et al.* 2011). Considering this fact, HSFA1 regulators, instead of the HSFA2, could directly regulate the *HSP25.3* expression in *hrr* mutant. Presumably, the obtained result could also be explained by different expression of this gene under these experimental conditions, plant development stage or even from the ecotype background where is expressed.

The HS-responsive gene expression levels assayed (*HSFA2, HSP18.1, HSP25.3, HSA32* and *HSP101*) suggest that HRR exerts important post-transcriptional regulatory functions over HS-specific genes. This regulatory action could explain, at last in part, the observed phenotype for mutant in basal thermotolerance assays (Section 3.2.3, Complementary Figure C3.5), though the developmental stage and tissues of phenotypic assays and expression analysis were different (16-days-old seedlings versus imbibed seeds). After HS treatment of wild-type *Ler* and *hrr* mutant imbibed seeds, the thermotolerance ability would be greatly determined by quantity and activity of HSPs and of their direct transcriptional regulators. Hence, the lowest levels of *HSFA2*, *HSP101* and, at minor extension, *HSP18.1* in *hrr* mutant during recovery time could predict the low thermotolerance and, consequently, low survival rate of corresponding seedlings.

In an attempt to further evaluate the relevance of HRR in regulation of HS-responsive transcripts, the transcript levels of *HSFA2*, *HSP101, HSP18.1, HSA32* and *HSP25.3* were evaluated in imbibed and HS-treated seeds of *HRR* over-expresssion lines (Figure 3.28). Under control

conditions, transcript levels of *HSFA2, HSP101, HSP25.3* and *HSA32* showed to be impaired in imbibed *hrr* mutant seeds, in comparison with wild-type *Ler* seeds. This result not only demonstrates the HSP and HSFA2 importance for quick response to HS treatment, as also may indicate the functional role of HRR in stability of their transcripts. Meanwhile, only *HSFA2, HSP101* and *HSP25.3* transcript levels were impaired in HS-treated *hrr* mutant seeds. In contrast, the *HSA32* and *HSP18.1* transcripts seemed to be more abundant in *hrr* mutant seeds than in wild-type *Ler* seeds under HS conditions. These results contrast with the previous expression analysis (Figure 3.27). This fact could be due to different experimental conditions and developmental stages used for both analyses.

In *HRR.1* over-expression seed lines, the expression of almost all HS-induced genes was up-regulated, with the exception of *HSP25.3*, whose transcript levels were drastically reduced in both lines. But, under control conditions, the JP9 seeds displayed the highest levels of other *HSP* and *HSFA2* transcripts. Under HS treatment, *HSP25.3* expression was slightly increased in JP9 line. Other differences between L2 and JP9 lines were detected. The *HSFA2* transcript levels were more elevated in JP9 seeds than in L2, as in control as under HS conditions. During HS treatment, L2 seeds exhibited a highest up-regulation of *HSA32*, in contrast to JP9 seed, that showed a reduction in its expression upon HS. The *HSP101* and *HSP18.1* expression levels were similar in both HRR over-expression lines and experimental conditions.

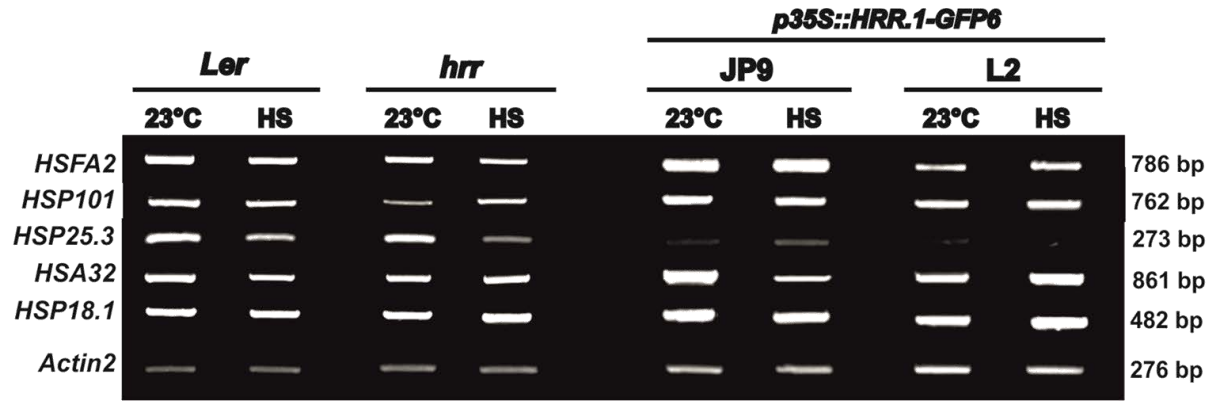


Figure 3.28 Expression analyses of HS-specific genes during heat stress imposition, in imbibed seeds, subjected or not to HS. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from imbibed wild-type *Ler*, *hrr* and HRR over-expression (JP9 and L2) mutant seeds, which were subjected to HS treatment (50ºC for 60 min) or maintained at standard conditions (23ºC). As internal controls, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are depicted in Annexes III and IV, respectively.

Altogether, these results suggest that, under standard conditions, the introduction of *HRR.1* over-expressing transgene promotes an accumulation of *HSP* transcripts. However, this is only valid when the transgene was introduced into the *hrr* mutant background (JP9 line), once the expression pattern in L2 seeds is more similar to wild-type *Ler*.

Under HS conditions, the expression levels of *HSP* and *HSFA2* transcripts in HRR over-expression lines quite predict the thermotolerance response developed in the previous germination assays (Figure 3.17). Comparing all seed lines under HS conditions, the JP9 seeds presented the highest levels of *HSFA2* transcripts (Figure 3.28). Under these conditions, the accumulation of *HSFA2* transcripts in JP9 seeds could suggest that HRR is important to promote the stability of *HSFA2* transcripts. Recently, the SUMOylation of HSFA2 was reported and suggested to repress the HSFA2 activity in recovery phase after HS. This repression leads to down-regulation of *HSP* gene expression and reduction of adquired thermotolerance (Cohen-Peer *et al.* 2010). Once verified a high accumulation of *HSFA2* transcripts in JP9 seeds and, probably high levels of HSFA2 protein, it is possible that other post-translational mechanisms could be involved in regulation of HSFA2 protein levels. Hence, this prediction corroborates with highest thermotolerance improvement of HRR over-expression seeds and seedlings (JP9). Indeed, previous studies suggested that the primary *HSFA2* transcripts are subjected to alternative splicing in response to the formation and accumulation of HS-misfolded protein aggregates, under HS conditions (Sugio *et al.* 2009). The positive ratio between full-length *HSFA2*/misspliced *HSFA2* transcripts could possibly culminate in a high maintenance of cellular homeostasis in JP9 seeds, under HS conditions, mainly promoted by RNA-binding proteins activities.

In conclusion, these results pointed for a crucial role of HRR in post-transcriptional regulation of HS-responsive transcripts (*HSPs* and *HSFs*), depending on the plant development stage. In seedlings, HRR could mainly function during recovery period, but also during the HS imposition. Indeed, HRR appear to be involved in stability of *HSFA2* and *HSP101* transcripts, during HS treatment and recovery periods. Also during recovery, HRR seems to be crucial to maintain the stability of *HSP18.1* transcripts. In imbibed seeds, HRR could be involved in regulation of the many of these HS-responsive genes (excepting HSP18.1). The high accumulation of *HSFA2* transcripts in HS-treated JP9 seeds could point for *HRR* function in stabilisation of transcripts corresponding to regulatory factors, such as the pivotal HSFA2. Ultimately, HSFA2 is important for induction of a specific set of genes (*HSPs*, in majority), promoting the re-establishment of cellular homeostasis under stressful conditions.

During germination the constant transcriptome and proteome remodelations would request the crucial function of chaperones in post-translational regulation. The presence of HSPs would be also essential for basal thermotolerance, promoting the quick reestablishment of protein homeostasis during recovery period. Therefore, the basal thermotolerance difference observed between wild-type *Ler* and *hrr* mutant (section 3.2.3) could be due to distinct expression levels of HSFs and HSPs, upon HS treatment conditions.

3.3.4.2 Seed-related genes

In silico gene expression predicted that *HRR* is up-regulated during seed development and germination processes (section 3.1.3). Moreover, the reduction of seed thermotolerance in *hrr* mutant seeds could result from the deregulation of seed development. During this process, HRR could eventually play post-transcriptional functions on transcriptome modulation, from early stages of embryogenesis (active cell division and morphogenesis) to seed maturation (compound accumulation and acquisition of desiccation tolerance).

The phase transitions occurring during the formation and maturation of embryo and during seed germination are supported by central transcriptional regulators that participate in the expression of downstream gene targets. These regulators include ABA-*insensitive* TFs (*ABI3, ABI4, ABI5*), *LEAFY COTYLEDON1 and 2* (*LEC1* and *LEC2,* respectively) and *FUSCA3 (FUS3)*. To understand if HRR exerts regulation function on the corresponding transcript levels during seed development, a semi-quantitative RT-PCR analysis was performed in wild-type *Ler* and *hrr* mutant (Figure 3.29A). During seed germination only the transcript levels of *ABI3*, *ABI4* and *ABI5* were analysed, due to their role on ABA signalling during post-germination process (Figure 3.29B). The other seed-specific regulator genes (*LEC1*, *LEC2* and *FUS3*), once presenting a embryo-restricted expression and being repressed by PKL during seed germination (Tiedemann *et al.* 2008), not were analysed during seed germination.

The seed-specific transcriptional regulators (*ABI3, ABI4, ABI5, LEC1, LEC2* and *FUS3*) (Figure 4.29A) presented high expression levels during seed maturation stages (4 throughout 6; Figure 3.29A). Although they do not appear to be significantly affected in *hrr* mutant, some slight fluctuations between the expression in wild-type *Ler* and *hrr* developing seeds were detected. *ABI5* transcript levels were slightly reduced in *hrr* mutant developing seeds, in stages 4 and 6 of seed maturation. Indeed, *ABI5* seems to be up-regulated in the first stages of seed development. A slight impairment of *LEC1*, *LEC2* and *FUS3* in *hrr* mutant was also detected, namely, the *LEC1* down-regulation in early stage of seed embryogenesis (stage 2) and the *LEC2* and *FUS3* down-regulation in stage 4.

ABI3, *ABI4* and *ABI5* transcriptional regulators presented high levels of transcripts in first day of germination, which are strongly reduced in the second day (Figure 3.29B). During germination, a difference between the *ABI* genes expression in wild-type *Ler* and *hrr* mutant was not detected, except for *ABI5*, whose transcript levels appeared to be slightly down-regulated in *hrr* mutant germinating seeds (Figure 3.29B).

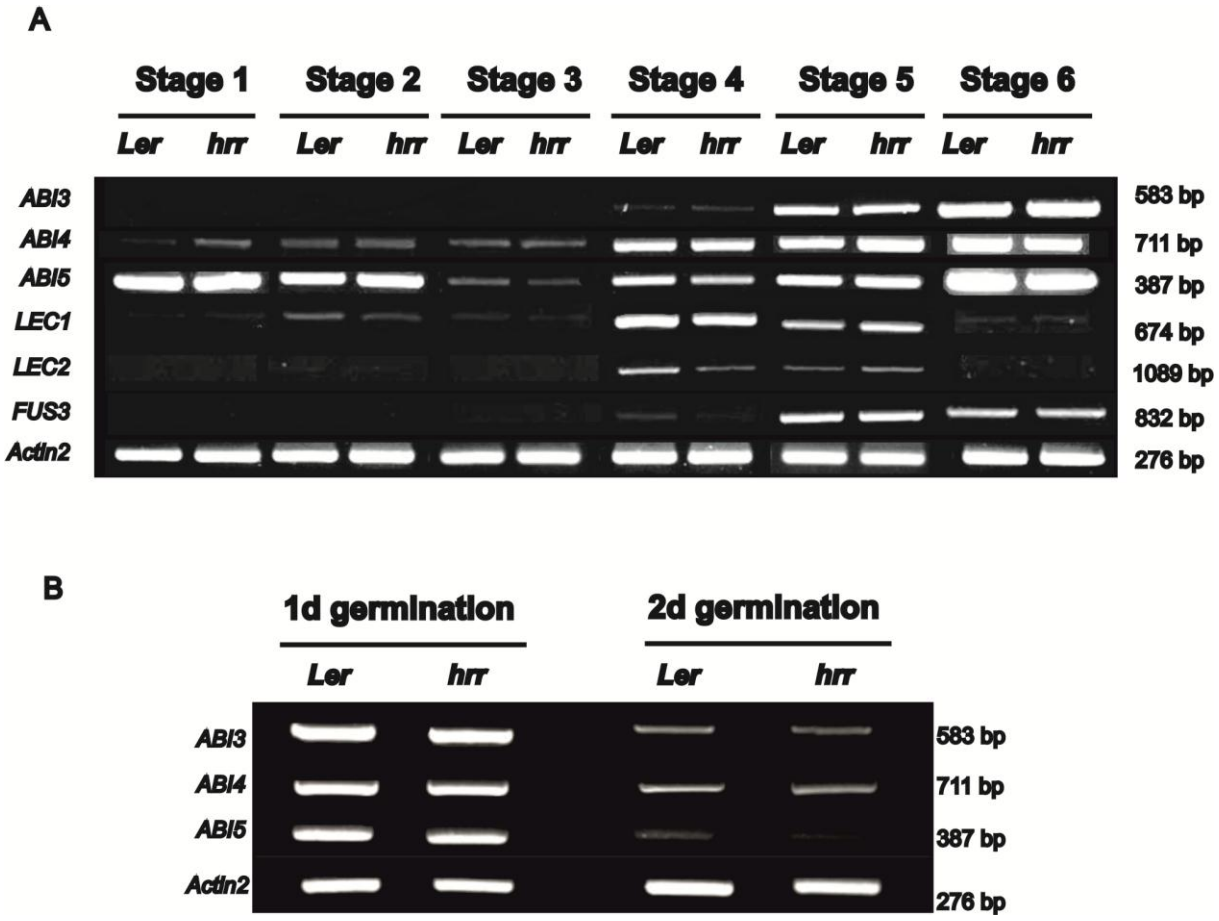


Figure 3.29 Expression analyses of key transcriptional regulator-coding genes during seed development and germination. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted (**A**) from flower/silique tissues, according to defined seed development and maturation stages (Figure 3.25) or (**B**) from germinated seeds with one or two days upon sowing in MS-agar medium. For comparison experiments were performed in wild-type *Ler* and *hrr* mutant. As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

The high expression of all transcription regulators evaluated (*ABI3, ABI4, ABI5, LEC1, LEC2* and *FUS3*) during seed maturation stages (stages 4 throughout 6) suggest their participation in induction of many genes coding for metabolic enzymes involved in accumulation of storage compounds (sugars, oil and seed storage proteins). This transcriptional induction is physiologically regulated by the increased levels of ABA during these stages.

ABI3 was described as a central B3 domain-containing regulator in signalling, being involved in seed regulatory programs, firstly in transition between embryo maturation and early seedling development (Nambara *et al.* 1995). Indeed, ABI3 possesses a structural domain (B1) involved in interaction with ABI5, modulating ABI5 activity (Nakamura *et al.* 2001). ABI4 is an AP2 transcription factor involved in sugar and ABA signalling. ABI4 controls its own expression and is essential in regulation glucose signalling during early seedling development (Bossi *et al.* 2009). In addition, ABI4

induces the *ABI5* expression during seed maturation, as well as induces the expression of specific plastid protein-coding genes (Bossi *et al.* 2009; Cutler *et al.* 2010). ABI5 is a bZIP and pivotal regulator in ABA signalling tighly regulated by post-translational mechanisms (phosphorylation, sumoylation and ubiquitination) and is involved in induction of *Em* genes during seed maturation stages (Stone *et al.* 2006; Miura *et al.* 2009; Cutler *et al.* 2010).

The highest transcript levels of these *ABI* factors in seed maturation stages (stages 4 throughout 6) could be related with higher ABA levels in these stages and accumulation of seed compounds. Exceptionally, ABI5 not only was expressed during seed maturation stages, but also presents expression during early stages of seed development. The detection of *ABI5* expression in these early stages could be due to the presence of floral tissue remains at these stages, in which *ABI5* expression has been reported (Schmid *et al.* 2005).

The high levels of *ABI3*, *ABI4* and *ABI5* transcripts, in the first germination day were suggested to be the result of the brief post-germination developmental arrest checkpoint mediated by ABA that occurs during seed imbibition (Lopez-Molina *et al.* 2001). Indeed, during early growth, following the seed stratification, a narrow developmental window where ABA regulates and stabilises endogenous ABI5 protein accumulation was suggested to occur (Lopez-Molina *et al.* 2001). In this work, 24h post-sowing, under light conditions, the *ABI* transcript levels remained high, being reduced at the second germination day, concomitant with increasing GA levels.

Only the *ABI5* expression seems to be impaired in *hrr* mutant during seed maturation and germination. Although no significant difference had been observed at the first day of germination, in second day it was perceptible the difference in stability of *ABI* transcripts in germinating *hrr* mutant seeds. The low levels of *ABI5* transcripts in *hrr* mutant seeds at later stage of seed maturation and in second day of germination suggested that HRR could regulate directly or indirectly the *ABI5* transcript levels. Although *HRR* transcript levels are low, as at later stage of seed maturation as at the first germination day (Figure 3.26), low amounts of HRR protein could be involved in the regulation of the *ABI5* transcripts stability. As ABI5 is a pivotal regulator in ABA signalling and is involved in induction of *Em* genes (LEA proteins) during seed maturation, the regulation of their transcripts is crucial to determine the accumulation of protective proteins (Carles *et al.* 2002). Ultimately, this regulation is essential for definition of longevity and resistance ability to environmental cues of *hrr* mutant seeds.

Other essential transcription factors are involved in seed development. *LEC1* gene encodes a homolog of the CCAAT-binding factor HAP3 subunit, while *LEC2* and *FUS3* are closely related to B3-containing protein transcription factors (Lotan *et al.* 1998; Luerssen *et al.* 1998; Stone *et al.* 2001). LEC1 and LEC2 transcription factors, which have been indicated as key regulators of

embryogenesis traits, they also promote the seed compound accumulation, simultaneously with FUS3 and ABI3, in early phases of seed maturation (Kagaya *et al.* 2005; Stone *et al.* 2008). FUS3, such as LEC1 and LEC2, is requested to the determination of cotyledonary cell identity and to the synthesis and accumulation of storage compounds (Tiedemann *et al.* 2008). Indeed, FUS3 promotes the dormancy and prevents precocious germination of immature seeds, by stimulating ABA synthesis while repressing GA biosynthesis (Chiu *et al.* 2012). The slight impairment of *LEC1* and *FUS3*, as well as *LEC2* at a more extension, in seed maturation (stages 4) observed in *hrr* mutant could suggest that HRR could be important for regulation of transcriptome during seed accumulation phases. Since *HRR* transcripts were not detected in the earliest stages of seed maturation (stages 1 and 2; Figure 3.25), the HRR function in regulation of *LEC* transcripts during this stage could not be predicted. However, due to weak sensitivity of semi-quantitative expression analysis for a minimal threshold of transcript, a real-time quantitative expression analysis should be performed to observe the *HRR* expression levels during early stages of embriogenesis. Hence, a better accuracy of HRR function in regulation of *LEC* transcripts could be made.

To evaluate the effect of *HRR.1* over-expression on *ABI* transcript levels, a semi-quantitative expression analysis was performed in imbibed seeds of wild-type *Ler*, *hrr* mutant and HRR over-expression lines, submitted or not to HS (Figure 3.30). Upon HS treatment, *ABI* transcripts appeared to be slightly up-regulated in *hrr* mutant, though no differences have been detected under control conditions. This result could indicate that HRR could interfere in regulation of *ABI* transcript levels, in ABA-dependent HS response. Indeed, the increased *ABI* transcript levels in *hrr* mutant are concomitant with the eventual increase of ABA content in these seeds, which ultimately culminates in germination retardation. In HRR over-expression lines, all the *ABI* genes were much more up-regulated, even under standard conditions. This up-regulation was more pronounced for *ABI5* gene, corroborating the previous suggestion that HRR could regulate positively the *ABI5* expression levels and thus acts as a positive regulator in ABA metabolism and signalling.

Together, these results suggest a possible direct or indirect participation of HRR in modulation of seed-specific transcripts during seed development and germination. This is particularly true, for *ABI5* transcripts, during later stage of seed maturation up to seed germination stages. Under HS conditions, HRR appears to play a role in the regulation of *ABI* transcript levels, which ultimately determine the thermoinhibition levels of seeds and their germinative ability.

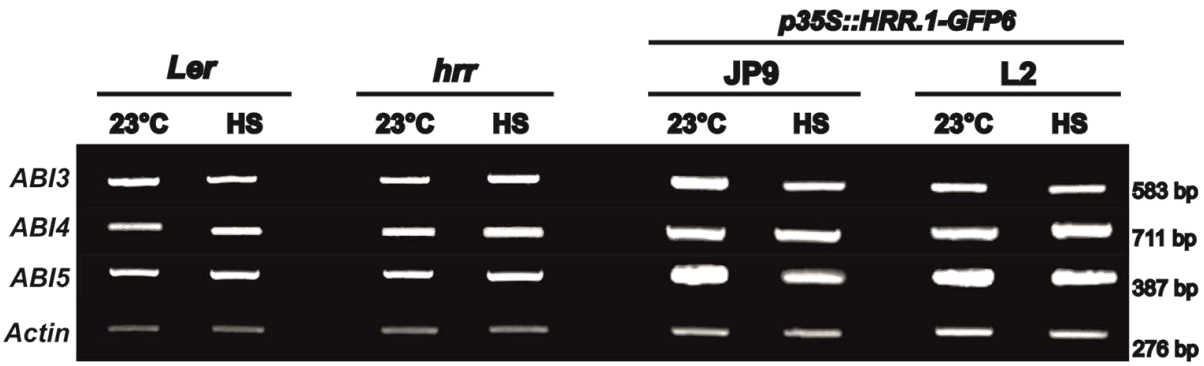


Figure 3.30 Expression analyses of *ABI* genes during heat stress imposition in imbibed seeds, subjected or not to HS. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from imbibed wild-type *Ler*, *hrr* and HRR over-expression (JP9 and L2) mutant seeds, which were subjected to HS (50ºC for 60 min) or were maintained at standard conditions (23ºC). As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are depicted in Annexes III and IV, respectively.

3.3.4.3 Stress-related genes

Besides the accumulation of storage proteins and lipids during seed maturation, stress-related proteins are also accumulated. LEA proteins and HSPs play essential protective functions during acquisition of dessication tolerance, at late maturation stage, preventing the macromolecules damage and promoting the maintenance of cellular stability. They are immobilised during early stages of seed germination, where some of them play protective roles and others are integrated in biosynthetic pathways. (Hong-Boa *et al.* 2005; Manfre *et al.* 2006; Kotak *et al.* 2007; Hundertmark and Hincha 2008; Manfre *et al.* 2009). As HRR is mainly expressed in later stage of seed maturation (stage 6, Figure 3.26A), it would be interesting and important to know if HRR is involved in regulation of transcript levels of some key stress-related proteins. For this, *Em1* and *Em6* (LEA proteins), *HSP101* and *HSFA9* gene expression levels were analysed during seed development (Figure 3.31A). Their corresponding genes display considerable expression levels during later stages of maturation (Kotak *et al.* 2007; Bentsink and Koornneef 2008). When the environmental conditions (light, nutrients and temperatures) are ideal, seed will enter in the germination process and the accumulated stress-related proteins will be recruited, allowing the osmotic adaptation of germinating seed. For this, the same transcript levels were analysed during seed germination (Figure 3.31B).

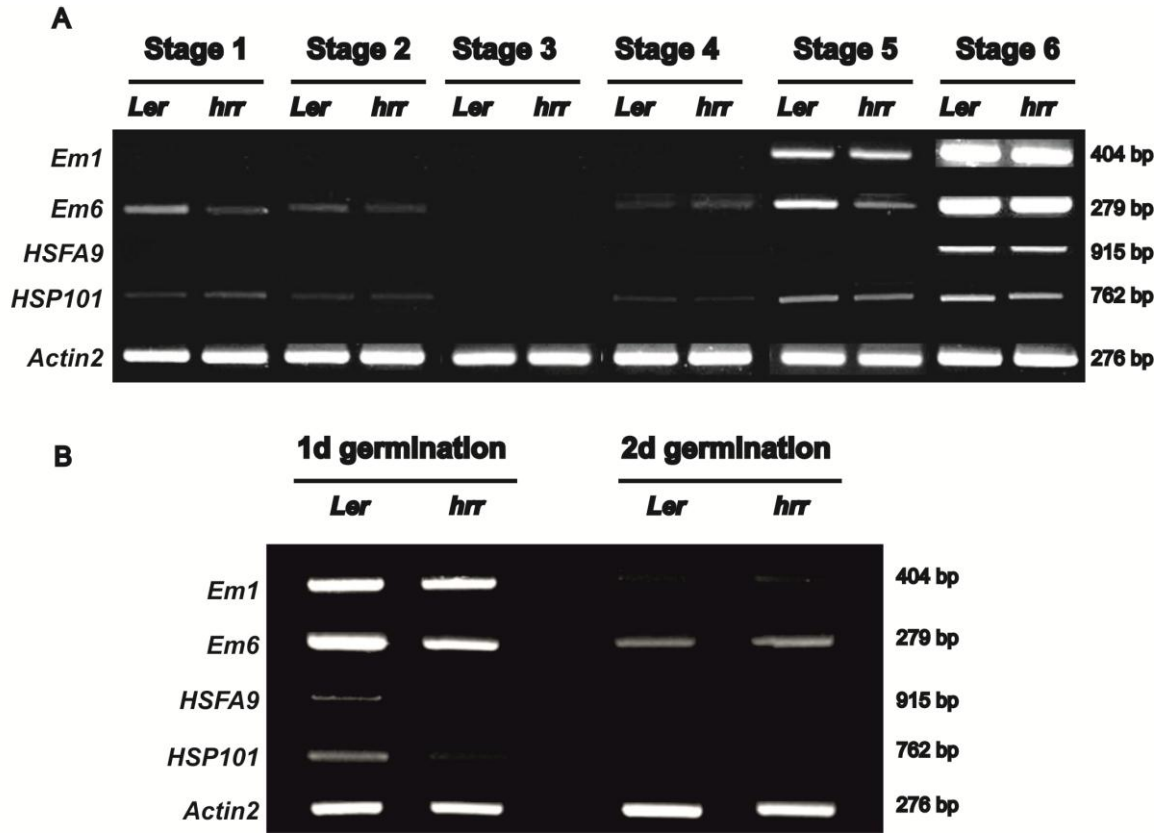


Figure 3.31 Expression analysis of LEA protein genes (*Em1*, *Em6*), *HSP* gene (*HSP101*) and seed-specific *HSF* gene (*HSFA9*) during seed development and germination. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted (**A**) from flower/silique tissues, according to defined seed development and maturation stages (Figure 3.25) or (**B**) from germinated seeds with one or two days upon sowing in MS-agar medium. For comparison experiments were performed in wild-type *Ler* and *hrr* mutant. As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

Em proteins belong to group 1 of Late Embriogenesis Abundant (LEA) proteins, being expressed in later stages of embryo maturation (acquisition of dessication tolerance) and during water deficit in vegetative organs, suggesting a protective role during water limitation (Hundertmark and Hincha 2008). HSFA9 was described as a specialised HSF for embryogenesis and seed maturation, controlled by hormonal networks (ABA and auxins) and involved in induction of *HSP* and *sHSP* promoters (Kotak *et al.* 2007; Carranco *et al.* 2010; Scharf *et al.* 2012). *HSP101* codes for a chaperone involved in protein remodelation through its ATPase activity (Singh and Grover 2010). This protein is not only implicated in Arabidopsis basal and acquired thermotolerance as it is regulated during seed development (Larkindale *et al.* 2007). HSP101 is accumulated during mid-maturation and stored in dry seed, in an expression pattern similar to that seen for LEA proteins and sHSPs (Xiong *et al.* 2001).

As expected, all stress-related transcripts were up-regulated in later stages of seed maturation (stages 5 and 6) (Figure 3.311A). In early stages of seed development (stages 1 and 2), only a

reduced expression was detected for *Em6* and *HSP101* genes. This could be due to the considerable expression of these genes in floral tissues as the samples harvested in the first stages of seed development contained remains of floral tissues (petals, stamens, pollen grains). Indeed, *in silico* data (e-FP browser, BAR) predicted that *HSP101* expression in carpels, stamens and petals. *Em6* was only predicted to be up-regulated in later stages of seed maturation and dry and imbibed (24 h) seeds. However, recent quantitative RT-PCR expression analysis data revealed that *Em6* is ubiquitously expressed in different Arabidopsis organs, displaying highest levels in seedlings, buds and flowers (Hundertmark and Hincha 2008).

In the first germination day, seeds presented high *Em1* and *Em6* expression levels that significantly declined in the second day of germination (Figure 3.31B). During the germination process, the higher levels of *Em* transcripts, in relation to *HSP101* and *HSFA9,* could be related with brief increased ABA levels during early phases of seed germination. This increase is crucial for environmental osmotic adaptation of germinating seeds, thus avoiding the damaging of important macromolecules. During germination, a much lower expression was observed for *HSP101* and *HSFA9* coding genes. .

The expression analysis revealed significant expression differences between wild-type *Ler* and *hrr* mutant seeds. During seed maturation and in first day of germination, *Em6* seems to be affected in developing *hrr* seeds (Figure 3.31A), while *HSP101* and *HSFA9* transcripts seem to be impaired in seeds during the first day of germination (Figure 3.31B). The expression impairment of these genes in *hrr* mutant suggests that HRR could be involved in the stability regulation of their transcripts. The expression regulation of *Em6* has been proposed to be performed by ABI factors, through the interaction/modulation of ABI5 with ABI3 (Nakamura *et al.* 2001; Carles *et al.* 2002). Recent studies indicated that ABI3 could also activate the *HSFA9* promoter, which in turn induces *HSP* promoters, such as *HSP101* (Kotak *et al.* 2007). In addition to a possible effect of HRR on stabilisation of *Em6*, *HSP101* and *HSFA9* transcripts, the transcriptional network between ABI factors, HSFA9, HSP101 and Em proteins could enhance the HRR effect on seed development.

Far studying the relevance of HRR on transcription of *Em1*, *Em6* and *HSP101* genes, a semi-quantitative expression analysis was performed in imbibed seeds (wild-type *Ler*, *hrr* mutant and HRR over-expression lines), submitted or not to HS treatment (Figure 3.32). The expression profiles of *Em* genes were quite similar in wild-type *Ler* and *hrr* mutant seeds and in both experimental conditions. Only a slight reduction of *HSP101* transcript levels was detected in *hrr* mutant seeds, in both experimental conditions. These results suggest that *Em* transcripts are not greatly affected by HS treatment (50ºC, during 60 min). Concerning the HRR over-expression lines, an up-regulation of all

genes was detected, after HS treatment and also in control conditions. This result seems to be a good indication of the importance of high levels of HRR protein for the stabilisation of seed-related proteins.

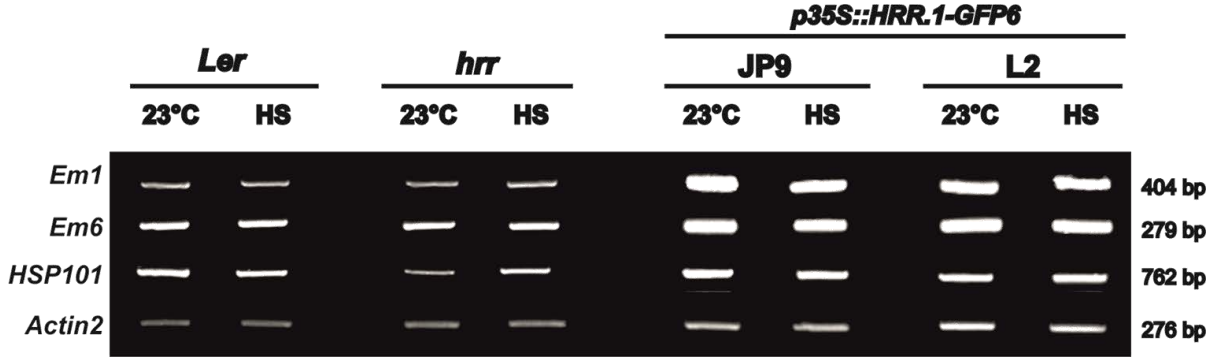


Figure 3.32 Expression analysis of LEA protein genes (*Em1, Em6*) and *HSP101* gene, in imbibed seeds, subjected or not to HS. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from imbibed wild-type *Ler*, *hrr* and HRR over-expression (JP9 and L2) mutant seeds, which were subjected to HS treatment (50ºC for 60 min) or were maintained at standard conditions (23ºC). As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair primers and PCR conditions are depicted in Annexes III and IV, respectively.

Altogether, the results showed that the evaluated stress-related genes (*Em1, Em6, HSFA9* and *HSP101*) are preferentially induced in later stages of seed development (stages 5 and 6) and in early stages of seed germination. Almost all genes (*Em6, HSP101* and *HSFA9*) appear to be regulated by HRR, once they were affected in *hrr* mutant in many of the developmental stages analysed. The up-regulation of *Em1*, *Em6* and *HSP101* in HRR over-expression line also supported this HRR role. The stability and integrity of stress-related transcripts would be crucial for seed development and germination, where dessication and osmotic stress conditions are stabilished.

3.3.2.4 ABA and GA metabolism genes

The seed development process is determined by hormonal regulation, not only by the ABA levels, but also by other phytohormone levels, such auxins, cytokinins and GAs (Toh *et al.* 2008). In particular, the development of seed maturation traits is determined by a tight balance between bioactive GAs and ABA. A feedback response mechanism controls the expression levels of rate-limiting enzymes involved in ABA and GA biosynthesis and catabolism, being thus dependent on the hormonal level fluctuations (Xiong and Zhu 2003; Sun 2008). For studying the possible involvement of HRR in post-transcriptional regulation of transcripts from ABA and GA metabolism components (*ABA1*, *NCED9*, *CYP707A1, GA3ox1* and *SPY*), a semi-quantitative expression analysis was performed in developing and germinating seeds of wild-type *Ler* and *hrr* mutant (Figure 3.33).

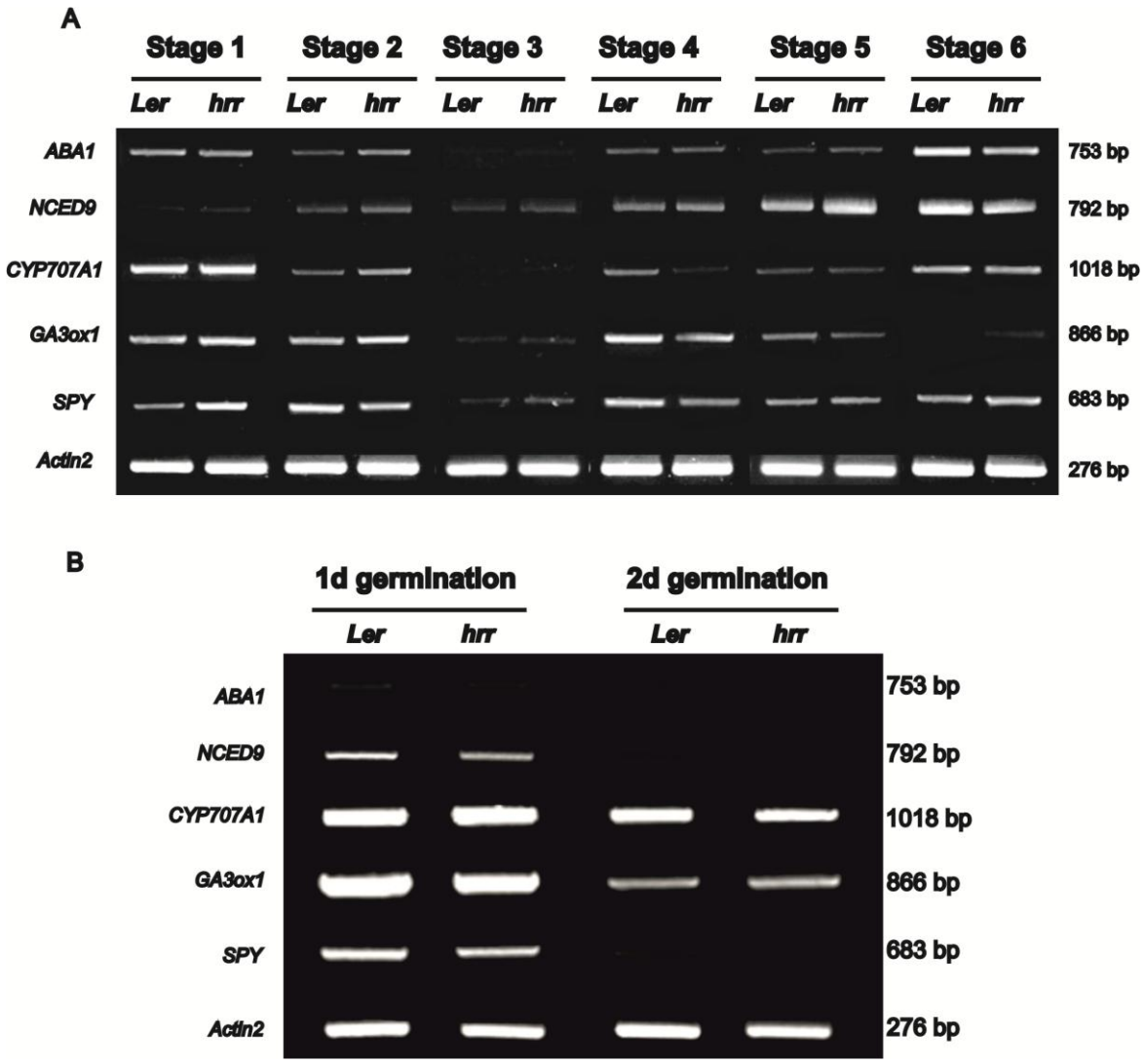


Figure 3.33 Expression analysis of ABA metabolism (*ABA1, NCED9*, *CYP707A1*) and GA metabolism (*GA3ox1*, *SPY*) related genes during seed development and germination. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted (**A**) from flower/silique tissues, according to defined seed development and maturation stages (Figure 3.25) or (**B**) from germinated seeds with one or two days upon sowing in MS-agar medium. For comparison experiments were performed in wild-type *Ler* and *hrr* mutant. As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

The importance of a fine tune regulation between ABA and GA hormones is revealed by ubiquitous expression of ABA- and GA-related proteins in all seed development stages (Figure 3.33A). *ABA1* (or *ZEP*), codes for a zeaxanthin epoxidase, which is involved in early steps of ABA biosynthesis, in plastids. *ABA1* is expressed in the embryo from globular to desiccation stages (Audran *et al.* 2001). *NCED9* belongs to a family of more four *NCED* genes (*NCED2, NCED3, NCED5, NCED6*), which codes for 9-cis-epoxycarotenoid dioxygenase (Xiong and Zhu 2003). This enzyme catalise the rate-limiting step in the ABA biosynthesis pathway, indicating its involvement in ABA biosynthesis regulation in seeds (Xiong and Zhu 2003). During seed development, *NCED9* is

expressed abundantly in immature seeds, in embryo and endosperm (Lefebvre *et al.* 2006). The ABA deactivation enzyme CYP707A1 corresponds to an ABA 8'-hydroxylase. Its coding gene is expressed during seed development, being also induced during post-germination growth (Kushiro *et al.* 2004; Okamoto *et al.* 2006).

Concerning ABA metabolism, the *CYP707A1* presented higher transcript levels in early stage of seed development than *ABA1* and *NCED9* ones. At late stage of seed maturation (stage 6) the *NCED9* and *ABA1* presented much higher levels, comparing with ABA-catabolic transcripts *CYP707A1*. During the seed development, the *ABA1* and *NCED9* transcripts are expressed during different stages of seed development (Xiong and Zhu 2003). The obtained results are in according with those previously suggested for *ABA1* and *NCED9* gene expression. Temporal increasing of *NCED9* transcripts was observed, whose highest levels culminate in the later stages of seed development (stages 5 and 6). It was also in stage 6 that *ABA1* transcripts reached the highest levels. Although the ABA action is predominant during the mid seed maturation stages (stages 4 and 5), ABA content decline to lowest levels in late stage of seed maturation. The highest expression of ABA biosynthesis genes in these maturation stages could be related with the synthesis of LEA proteins. Between mid- and late-maturation stages occurs ABA accumulation, that induces LEA protein genes, preparing the embryo for desiccation (Xiong and Zhu 2003). The expression profile of *CYP707A1* in seed development and germination is coincident with the crucial seed transition phases, where the ABA levels need to be strictly regulated (Yamaguchi *et al.* 2007). The up-regulation of this gene in early stages of seed development (stage 1) allows the regulation of ABA levels in a development stage where the high levels of GA are essential for embryo growth (Finkelstein 2010). During seed maturation stages, the levels become reduced, which is coincident with the increasing levels of ABA crucial for seed maturation and desiccation.

GA3ox1 genes codes for the GA3-oxidase, which catalises the conversion of an intermediate GA compound (GA_9) in a bioactive gibberellin (GA_4) (Sun 2008). *GA3ox1* is transiently induced during early embryogenesis and highly expressed in seeds imbibed in light (Mitchum *et al.* 2006). *SPY* codes for a protein with significant similarity to O-linked GlcNAc transferase (OGT) from animals (Tseng *et al.* 2001). SPY has been suggested as a negative regulator of GA signalling, once the knockout of the *SPY* gene leads to elevated GA responses (Swain *et al.* 2001). This transferase promotes the post-translational modification (addition of GlcNAc monossacharide) of components of GA signalling pathway (Qin *et al.* 2011). In case of GA-related genes, *GA3ox1* and *SPY* were expressed during early embriogenesis stages. The *GA30x1* transcript levels decreased during later stages of seed maturation (stages 5 and 6), whereas the *SPY* transcript levels leaned to increase. Then, the *GA3ox1* transcript levels drop during seed maturation stages (stages 5 and 6), coincident

with the increasing levels of ABA. The *SPY* expression profile during seed development shares some similarities with *ABA1* and *NCED9* expression profiles. In addition of its negative regulatory roles in GA metabolism and signalling, SPY appears to be an activator of other hormonal signalling pathways (Olszewski *et al*. 2002). The *SPY* expression profile could thus indicate the importance of this regulator in the controlling GA levels during seed development, in those stages where ABA is synthesised. For this instance, an interaction between SPY and some components of ABA metabolism and signalling could be then predicted (Lovegrove and Hooley 2000).

During germination process, the ABA-related genes, *ABA1* and *NCED9* presented less expression during seed germination, being *ABA1* transcript levels almost undetectable (Figure 3.33B). In contrast, the gene expression of *CYP707A1* was highly up-regulated after the first day of germination, exhibiting a slight decrease afterwards. A similar expression profile was detected for *GA3ox1*, though presenting lower transcript levels at the second day. As observed for seed maturation, the *SPY* expression profile was similar to those for *ABA1* and *NCED9*. The *SPY* transcript levels were also expressed at the first day of germination and were abruptly reduced at second day. These results corroborate with GA and ABA crosstalk during germination process. In the early stages of germination occurs a transient increasing of ABA levels, which promotes the osmotic adaptation of new seedling (Lopez-Molina *et al.* 2001). For this, it is observed an increasing of *NCED9* expression levels in first day of germination. With raising levels of GA, occurs an increasing in expression of ABA catabolic *CYP707A1* and GA biosynthetic *GA3ox1* genes.

The expression analysis revealed some expression differences between wild-type *Ler* and *hrr* mutant. *ABA1, NCED9* and *SPY* expression levels were slighty impaired in *hrr* mutant, during seed germination (Figure 3.33B). This suggests that HRR could be involved in regulation of these transcripts during transition phases from seed dessication to seed germination. Hence, these results suggest that HRR could be involved in stability regulation of ABA biosynthesis (*ABA1* and *NCED9*) and *SPY* transcript levels, whose proteins are involved in crucial rate-limiting reactions of ABA and GA metabolism (Xiong and Zhu 2003).

To study the effect of HRR on the expression of the same ABA- and GA-related genes, a similar analysis was performed in imbibed seeds from wild-type *Ler*, *hrr* mutant and HRR over-expression lines, either submitted or not to HS (Figure 3.34). When comparing the expression of wild-type *Ler* and *hrr* mutant seeds, only *CYP707A1* and *SPY* presented a slight impairment in *hrr* mutant seeds, under control conditions. However, all analysed ABA and GA metabolism-related genes were up-regulated in HRR over-expression lines. An up-regulation of *ABA1*, *GA3ox1* and *SPY* genes was observed in JP9 seeds, in relation to L2 seeds. However, when subjected to HS treatment, mainly the *ABA1* and *SPY* transcript levels were higher in L2 seeds than in JP9 seeds.

These results suggest the importance of HRR accumulation in regulation of ABA biosynthesis and *SPY* transcript levels during seed thermoinhibition phenomenon. This mechanism is characterised by temperature-induced accumulation of ABA levels in seeds, which delays the germination and plays a protective role at high temperature (Toh *et al.* 2008). Hence, the control of ABA biosynthesis and GA negative regulator transcript levels by HRR protein under high temperatures could be fundamental for further seed germination viability, during recovery period.

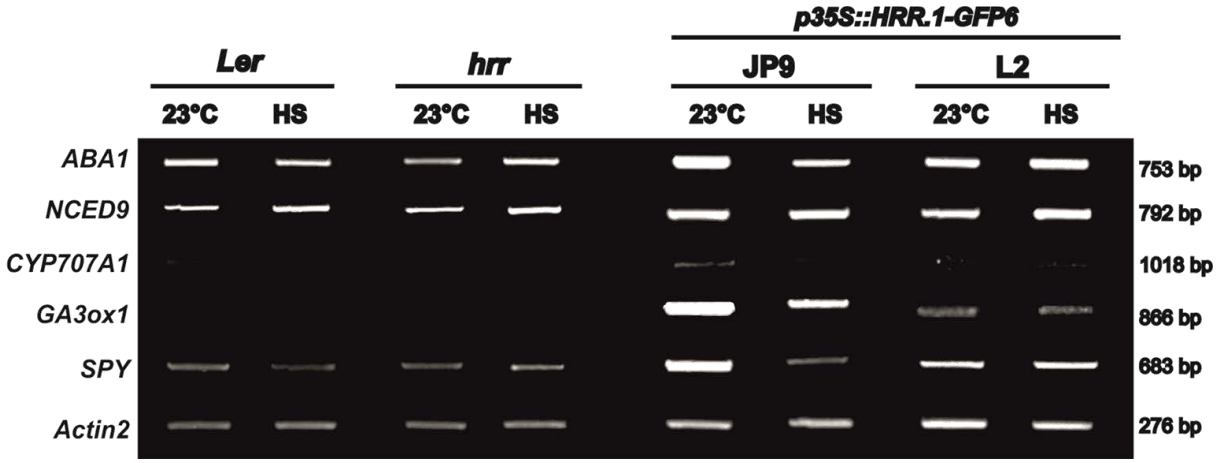


Figure 3.34 Expression analyses ABA metabolism (*ABA1, NCED9* and *CYP707A1*) and GA metabolism (*GA3ox1* and *SPY*) related genes, in imbibed seeds, subjected or not to HS. Transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from imbibed wild-type *Ler*, *hrr* and HRR over-expression (JP9 and L2) mutant seeds, which were subjected to HS treatment (50ºC for 60 min) or were maintained at standard conditions (23ºC). As internal control, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair primers and PCR conditions are depicted in Annexes III and IV, respectively.

Globally, these results suggest that HRR could be involved in stability regulation of key transcripts associated to ABA and GA metabolism. This might be depicted when in first seed germination day, the ABA metabolism (*ABA1* and *NCED9*) and *SPY* transcripts were down-regulated in *hrr* mutant. Due to hormonal adjustments during seed germination, the expression of ABA and GA metabolism genes is extremely regulated. Hence, the regulated turnover of these transcripts becomes crucial for transcriptome remodelation during germination, where HRR could be part. Moreover, during imposition of HS, HRR could play some function in control of the positive feedback of ABA biosynthesis and in negative regulation of GA synthesis. Further, this reflects in resistance to thermoinhibition and germination ability of Arabidopsis seeds.

3.3.5 mRNA decay analysis of alternative-spliced *HRR* transcripts

As previously discussed, an alternative transcript of *HRR* was detected in seedlings (*HRR.2*), under HS conditions (section 3.3.1). This transcript harbours the first intron sequence and results from an

intron retention process. Consequently, *HRR.2* possesses an in-frame PTC and was suggested to be a potential target for NMD. Depending from developmental and/or environmental signal input, the remodelation of transcriptome could be greatly affected by drastic alterations at level of pre-mRNA processing, producing many alternative transcripts, which could harbour PTC**.** This sort of transcripts normally are usually considered potential targets for NMD, which has been described as being the principal control system of aberrant transcripts and mRNA turnover (Shyu *et al.* 2008). NMD is one of the mRNA surveillance used by eukaryotic cells to control the quality of mRNA function, by eliminating abnormal transcripts (Maquat 2004). In recent years, some mechanism of NMD have been proposed in plants, mostly based in mammallian and yeast models. The most attractive model is based in distance between the PTC and other sequences that are usually present within the 3'UTR (Kerényi *et al.* 2008). If they are too far from the PTC, the PTC-containing transcript would be driven for NMD. The NMD is triggered by a core of *trans* factors (UPF1, UPF2, UPF3) that, together with the exon junction complex (EJC), bind to aberrant transcripts and eventually move them to degradation (Hoof and Green 2006).

For understanding the decay mechanism of *HRR.2*, an analysis of mRNA half-life of *HRR* alternative transcripts was performed, making use of transcription and translation inhibition treatments with actinomycin D (ActD) and cycloheximide (CHX), respectively. ActD binds to DNA and inhibits the elongation executed by RNA polymerase. The use of ActD allows to determine if the levels of transcripts are only dependent of gene transcription activity. CHX is a translational inhibitor that interferes with the peptidyl transferase activity of 60S ribosomal subunit, promoting the stabilisation of polyribosomes-RNA complexes (Anderson and Kedersha 2002; Hori and Watanabe 2008). Since NMD is considered a translation-dependent pathway, the use of CHX allows the evaluation of mRNA degradation-dependent on polyribosome release. ActD and CHX treatments were performed just after imposition of HS treatment (38ºC during 60 min) to wild-type *Ler* seedlings (16-days-old). For comparation, the same inhibition treatments were performed in seedlings at standard conditions (23ºC). Control samples corresponded to *HRR* expression profile in wild-type *Ler* seedlings under standard growth conditions (23ºC) and under HS treatment (38ºC, 60 min) (Figure 3.35).

When wild-type *Ler* seedlings were grown at standard conditions (23ºC), no expression of *HRR* occurred (Figure 3.35). However, heat-stressed (38ºC, 60 min) seedlings exhibited high levels of *HRR.1* and *HRR.2* transcripts, though a greater amount of *HRR.2* transcript was evident. These results were similar to those previously observed (Figure 3.22). When seedlings growing in standard conditions were treated with ActD or CHX, none or basal expression of *HRR* gene was observed (Figure 3.35). The immediate ActD treament (after HS) promoted the reduction of *HRR.2* transcript levels, in relation to HRR.1. However, *HRR.1* transcript levels upon ActD treatment are very similar

to those upon HS condition. The constant *HRR.1* transcript levels suggest its increased stability. Moreover, as the application of ActD interrupts the production of transcripts, the results suggest that the regulation of *HRR.2* transcript levels is mostly determined by transcription rate of *HRR* gene. Upon CHX treatment, heat-stressed seedlings presented an up-regulation of *HRR*, presenting much higher *HRR.1* transcript levels than *HRR.2* (Figure 3.35). This result suggests an even higher stabilisation of *HRR.1* transcripts upon CHX treatment, due to the CHX role in freezing of transcripts into polyribosomes (Anderson and Kedersha 2002). As *HRR.2* transcripts were not stabilised as *HRR.1* transcripts in polyribosomes, a NMD process would be important for *HRR.2* transcript decay. While *HRR.2* transcripts could be driven for degradation through nuclear and cytoplasmic NMD mechanisms, the *HRR.1* transcripts are maintained under steady-state conditions.

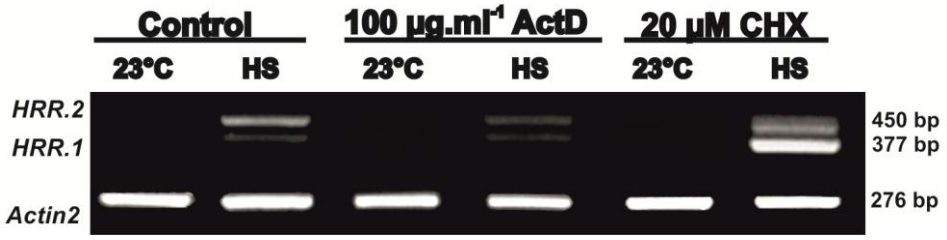


Figure 3.35 Evaluation of alternative spliced *HRR* transcripts decay, after heat stress imposition. *HRR.1* and *HRR.2* transcript levels were evaluated by semi-quantitative RT-PCR from mRNA extracted from treated wild-type *Ler* seedlings with 16-days-old. After HS treatment (38ºC, 60 min), seedlings were treated with either 100 µg.ml^{-1} actinomycin D (ActD) or 20 µM cycloheximide (CHX). Controls were prepared in the same way but without inhibitor supplementation. For comparation, similar treatments were performed in non-heat treated seedlings (23ºC). As internal controls, the transcript levels of *Actin2* (*Act2*) were analysed. Numbers on the right correspond to the expected sizes of PCR products. The pair of primers and PCR conditions are described in Annexes III and IV, respectively.

Considering some homology between human and plant processes, it is likely that CHX might influence the transcription and improve the stability of *HRR.1* transcripts, under HS conditions. Some studies in animal cells reported that the CHX treatment could lead to the superinduction and high stabilisation of *IL-6* mRNA levels and IL-6 accumulation (Hershko *et al.* 2004). This suggests that protein synthesis inhibition do not seems to be exclusively a inducer of protein and transcript degradation mechanisms. Regarding this, it is likely that CHX treatment could induce the transcription of *HRR* and increase the levels of *HRR.1* transcripts under HS conditions, possibly through prolongated activation of signalling components and stress-related TFs.

The alternative-spliced *HRR.2* transcripts are unstable molecules due to the presence of the PTC that, in turn, could provide a sort of cellular toxicity. For this reason, *HRR.2* transcripts could bind to specific nuclear proteins that drive them to nuclear mRNA surveillance processes. Recently, Kim *et al.* (2009) described that UPF2 and UPF3 bind to plant aberrant transcripts, accumulating them in the nucleolus where they will be probably processed through a NMD-like mechanism. Once

in cytoplasm, the recognition of PTC in *HRR.2* could be due to the fact of EJC still bound *HRR.2* transcripts (close to last exon-exon junction and downstream of PTC) and avoid the recognition of *cis* elements in 3'UTR, when occur the premature termination of translation. Then, the association of NMD factors (namely the phosphoregulated UPF1, together with UPF2 and UPF3) with premature terminating ribosome is postulated to facilitate the recruitment of decapping and degradation factors (Amrani *et al.* 2006; Schwartz *et al.* 2006; Kerényi *et al.* 2008).

Globally, the results suggest that *HRR.2* is a potential target for NMD or other RNA degradation mechanism. Although a more accurate assay would be necessary mRNA half-life of both transcripts, the preliminary results using ActD and CHX suggest that *HRR.2* transcript is driven for degradation. *HRR.1* transcripts appeared to be more stable than *HRR.2* transcripts. Hence, the recruitment of mRNA degradation machinery to remove *HRR.2* transcripts resulting from inaccuracy of pre-mRNA processing mechanism seems to be important for HS responses.

3.3.6 Histochemical analysis of *HRR*

The β-glucuronidase gene (*gusA*) has been extensively used as a reporter gene for histochemical analysis of gene expression. To access in which organs/tissues the *HRR* expression occurs, the predicted *HRR* promoter sequence (p*HRR*) was fused to the *gusA* gene. The resulting transgenic plants (wild-type *Ler* background) were used to detect the *GUS* expression occurring by *HRR* promoter activation. GUS activity was evaluated by the observation of a blue signal, resulting from the degradation of the GUS specific substrate (X-Gluc), thus indicating the *HRR* expression location. The GUS assay was performed either on ectopic GUS (p*35S::GUS*) or p*HRR::GUS* transgenic seedlings (seven-days-old). The p*HRR::GUS* seedlings were heat-stressed (38ºC, three hours), such as wild-type *Ler* ones, whereas the p*35S::GUS* were not heat-stressed but maintained at control conditions (23ºC).

Only the transgenic seedlings ectopically-expressing *GUS* have developed a blue signal, resulting from GUS activity over the substrate X-Gluc. When p*HRR::GUS* transgenic seedlings were submitted to HS treatment, no GUS signal was detected. The same result was obtained using different independent p*HRR::GUS* transgenic lines, as well as using wild-type *Ler* seedlings samples. As expected, non-heat-stressed samples, both wild-type *Ler* and p*HRR::GUS* transgenic seedlings, did not also exhibit GUS signal (data not shown).

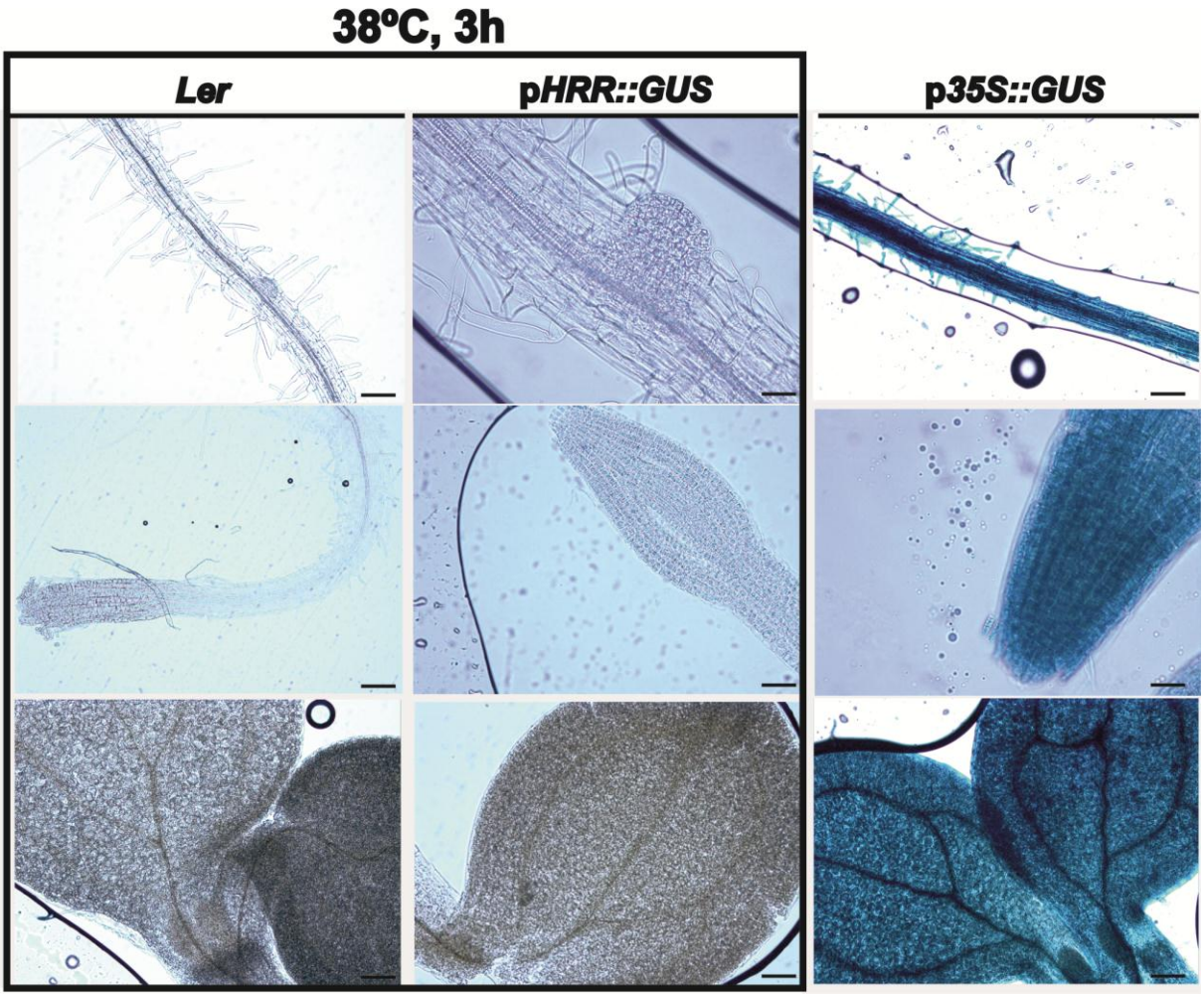


Figure 3.36 Histochemical localisation of *HRR* promoter activity in Arabidopsis seedling tissues. Transgenic (in *Ler* background) seven-days-old seedlings, expressing the p*HRR::GUS* fusion were subjected to the GUS assay (37ºC, overnight). As control, wild-type *Ler* seedlings were analysed in similar conditions. As positive control, transgenic seedlings harbouring the p*35S::GUS* fusion were directly subjected to GUS assay (without heat treatment, 23ºC). The blue signal indicates the *GUS* expression driven by the *HRR* promoter or by the constitutive *CaMV 35S* promoter (p*35S*). Under constitutive expression, *GUS* expression is observed in all tissues. None *HRR* promoter activity is observed after heat-stress treatment, being the signal similar to that wild-type *Ler*, under same experimental conditions. Scale: 0.5 cm.

Although the *HRR* expression, under HS conditions, is relatively low when compared with other HS-responsive genes, the absence of an evident GUS signal is questionable. At least, some *HRR* promoter activity would be expected to be detected in roots of p*HRR::GUS* transgenic seedlings. However, even with HS treatment, no GUS signal was detected (Figure 3.36). The presence of *cis*-elements in *HRR* exons and introns could be necessary for induction of *HRR* promoter. Recent reports have described the existence of *cis*-acting elements in intron sequences that could promote the gene expression enhancement (Parra *et al.* 2011). These enhancing introns are tipically located within the transcribed sequences, near the 5' end of the transcript and are compositionally distinct from downstream introns. These effectors of intron-mediated enhancement (IME) mechanisms could appear in 5'UTRs or in the coding regions near the transcription start site. If the *HRR* promoter

regulation is enhanced by a similar mechanism, the absence of intronic and/or exonic *cis*-acting elements in the transgene would promote a reduction on *HRR* promoter activity. The high expression of *HRR* gene could be possibly assisted by combinatorial influence of both types of *cis*-elements. Ultimately, this influences all transcriptional machinery, as well as subsequent post-transcriptional and translation mechanisms. The absence of GUS activity could be also due to the production of the *gusA* primary transcripts with unfavourable conformations, thus compromising downstream mechanisms, such as mRNA processing and translation processes.

Taken together, these results suggest that *HRR* is highly expressed during HS imposition, corroborating the predicted bioinformatic data (section 3.1). Moreover, HRR seems to be involved in the stability regulation of a specific set of transcripts induced during HS conditions, as well as during seed maturation and germination.

In contrast to the predicted bioinformatic data, under experimental HS conditions, *HRR* expression was subjected to an alternative splicing process. Hence, two alternatively-spliced *HRR* transcripts were produced: the canonical *HRR.1* and the intron-retained transcript *HRR.2*. However, in HS-treated seeds, only *HRR.1* transcript was expressed. By possessing a PTC, *HRR.2* transcripts were suggested to be a potential target of NMD. Indeed, the results obtained from mRNA decay analysis suggest that *HRR.2* transcripts are driven for degradation, possibly through NMD mechanism. The fast removing of these aberrant transcripts is fundamental to avoid cellular toxicity (imposed by accumulation of aberrant transcripts and truncated proteins) and prolonged homeostasis imbalance during thermotolerance responses.

Under HS conditions and in specific plant development processes, HRR could be crucial for the regulation of HS-responsive *HSF* and *HSP* transcripts. Particularly, HRR appears to be involved in the stability of *HSFA2* transcripts during HS imposition and recovery periods. Considering that HSFA2 is a pivotal and amplifier factor in induction of other *HSF* and numerous *HSP* genes, the tight control of *HSFA2* expression levels is extremely important under HS conditions. The possible involvement of HRR in the stability of *HSFA2* transcripts could be crucial for an adequate thermotolerance response, which could depend on intensity and duration of HS input.

In addition to the direct HS-induced genes (*HSFs* and *HSPs*), HS also affects the hormonal homeostasis. HRR appears to play a function in the regulation of ABA biosynthesis (*ABA1* and *NCED9*) and signalling (*ABIs*) transcripts, as well as *SPY* (GA negative regulator) transcripts upon HS treatment. The stability regulation of such transcripts is ultimately reflected in the seed thermoinhibition level under HS conditions, which further reveals the seed germination ability in recovery period. Also, during seed germination, in addition to *ABI5* regulation of transcript levels,

HRR appears to be involved in the stability regulation of ABA biosynthesis (*ABA1* and *NCED9*) and *SPY* transcripts. The regulation of ABA-related transcripts also reinforces the premise that HRR could be a positive regulator in ABA metabolism and signalling. As seed germination is mainly determined by hormonal balance between ABA and GA levels, the regulation of expression levels of ABA metabolism-related genes, particularly those coding for rate-limiting enzymes (NCEDs), is important to control the positive feedback pathway in ABA biosynthesis.

During plant development, *HRR* was expressed in the later stages of seed maturation, seed imbibition and germination (first day). This result suggests that HRR could regulate transcriptomes associated to seed maturation, desiccation and germination programmes. Consistent with *HRR* expression profile, the expression analyses of *ABI5, Em6, HSFA9* and *HSP101* revealed that these transcripts could be regulated by HRR, during later seed maturation and in first day of germination. In these stages, where an increasing of ABA levels also occurs, ABI5 acts as master regulator in ABA signalling, being the main responsible for the induction of *Em* genes. The correct accumulation of Em proteins (and other LEA proteins) and HSPs during seed maturation ultimately determines the seed longevity and post-embryonic development. In addition, HSFA9 is the unique HSF involved in seed expression regulation of *HSP* and *sHSP* genes, particularly *HSP101*. Thus, considering the transcriptional network between *ABI5, Em6, HSFA9* and *HSP101*, the stabilisation and integrity of respective transcripts promoted by HRR could be important to overcome the stress osmotic conditions during seed desiccation and germination.

Regarding these facts, HRR could be a determinant RNA-binding protein involved in the stabilisation and accumulation of transcript levels of pivotal regulators (HSFA2, ABI5) and rate-limiting enzymes (NCED9). Ultimately, the regulation of these transcript levels could influence the downstream gene expression, under HS conditions and during seed development and germination.

3.4. Subcellular dynamics of HRR proteins: perspectives on functional roles

For predicting the possible function of HRR.1 and HRR.2, through the understanding of their possible cellular target, the subcellular localisation of HRR.1 and HRR.2 proteins was determined using different approaches. The transient ectopic expression of HRR.1 and HRR.2 fusions (either N- or C-terminal to protein) was performed into epidermial cells of *Nicotiana benthamiana*. This allowed at first instance to analyse which are the most probable subcellular compartments where these proteins could be targeted. The stable transformation on cell suspension cultures of *Nicotiana tabacum* BY2 (Bright-Yellow 2) was performed using the same GFP fusion constructs under the control of *HRR* promoter (p*HRR*). Using BY2 cell cultures, a more versatile experimental system was designed for studying the effects of HS and chemical treatments on subcellular localisation of HRR proteins. Even lossing or modifying some functional mechanisms due to cellular undifferentiation, these cell cultures respond to diverse signals in a physiological manner similar to the responses observed in whole plant.

3.4.1 Subcellular localisation of over-expressed HRR.1 and HRR.2 proteins

For determining the subcellular localisation of HRR proteins, epidermial cells of *N. benthamiana* were transformed with *HRR.1* and *HRR.2* GFP6 ectopic transgenes. The transient over-expression of GFP6-HRR.1 and GFP6-HRR.2 (N-termini) and HRR.1-GFP6 and HRR.2-GFP6 (C-termini) fusions was evaluated two days after transformation (Figure 3.37).

The results for GFP6-HRR.1 protein fusion was uniformly detected in the nucleoplasm and associated to a sort of cytoplasmic network, which seems to be cytoplasmic granules associated to cytoskeleton (Figure 3.37A, solid and dashed red arrows, respectively). This fusion was also evident in the sub-nuclear region, most likely the nucleolus (Figure 3.37B, orange arrow). The GFP6-HRR.2 fusion was detected in small cytoplasmic granules, less intense than for GFP6-HRR.1 fusion (Figure 3.37E). The most intensive signal was detected in the nucleus, as nuclear speckles (C and D). These nuclear speckles were found either distributed throughout the nucleoplasm (Figure 3.37C) and/or localised close to the nuclear periphery (white asterisks, Figure 3.37D). The C-terminal HRR.1 and HRR.2 fusions (HRR.1-GFP6 and HRR.2-GFP6, respectively) displayed different GFP6 signals comparing to the corresponding N-terminal fusions (Figure 3.37F and H). The HRR.1-GFP6 fusion

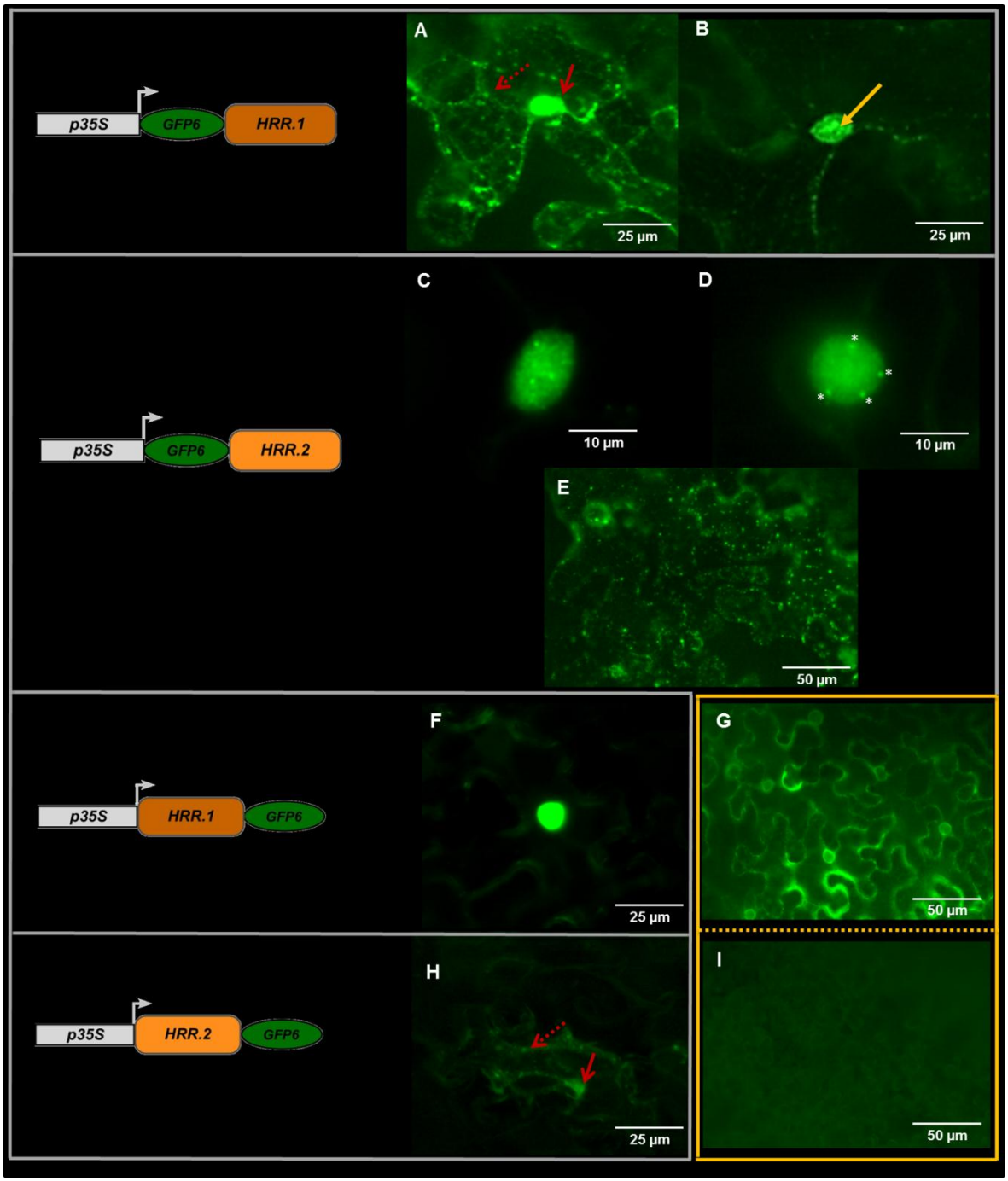


Figure 3.37. Subcellular localisation of ectopic HRR.1- and HRR.2 fusions in *Nicotiana benthamiana* epidermal cells. Transient expression of *p35S::GFP6-HRR.1/HRR.2* and *p35S::HRR.1/HRR.2-GFP6* transgenes was performed in leaf epidermial cells of *N. benthamiana*. Shematic representations of the fusion constructs used in each assay are displayed on the left. Images were obtained two days after transformation, using a fluorescence microscope. Different expression patterns of GFP6-HRR.1 (**A-B**), GFP6-HRR.2 (**C-E**), HRR.1-GFP6 (**F**) and HRR.2-GFP6 (**H**) fusions are depicted. The transient expression of p*35S::GFP* (pBIN) was performed in the same experimental conditions, being used as positive control (**G**). As negative control, non-transformed *N. benthamiana* epidermial cells were used (**I**). The * indicate nuclear speckles; solid red arrow indicates nucleus; dashed red arrow indicates cytoplasmic granules; orange arrow represents the nucleolus.

presented the majority of GFP6 signal in the nucleus, which was uniformly detected in nucleoplasm (Figure 3.37F). The GFP6 signal of HRR.2-GFP6 fusion was considerably weak, being only fairly detected in cytoplasmic granules and nucleus (Figure 3.37H). All N- or C-terminal fusion signals were significantly different from over-expressed GFP in control (p*35S::GFP*), where the typical distribution of GFP6 signal throughout the whole intracellular compartments was evident (Figure 3.37G).

Different subcellular patterns detected for each GFP6 fusion could result from the effect of constitutive promoter activity in global cellular homeostasis. The presence/absence of functional domain in HRR proteins and different terminal GFP6 fusions could also have resulted in different expression patterns. A comparative work of C- and N-terminal GFP fusions for subcellular localisation studies demonstrated that all tested C-terminal tagged proteins localised correctly, in contrast to N-terminal tagged proteins (Palmer and Freeman 2004). Accordingly, the authors suggested that N-terminal tagging of a protein can cause the targeting sequence to be masked. As proteins first emerge from the ribosome into the cytoplasm by the N-terminus and chaperones prevent their folding until a whole protein domain is exposed (50-300 amino acids long), the GFP6 will be firstly folded in N-terminal fusions. This could culminate in disruption of the correct folding of the protein of interest and its correct localisation. Also, the protein of interest may disrupt the folding of the GFP6 itself. In case of C-terminal fusions, as the folding of GFP6 occurs at the end, it will not influence the native folding of protein and the functional features of the protein of interest are expected to be maintained.

The different intracellular targeting of HRR.1 and HRR.2 could result in part from the interference of GFP6 tagging in the activity of predicted HRR domains, RRM and PABP-1234. Both N-terminal GFP6 fusions could interfere with the RRM domain activity, eventually affecting the binding to RNA molecules or interaction with other factors. As both N-terminal fusions (GFP6-HRR.1 and GFP6-HRR.2) were detected in apparently uniform cytoplasmic granules (in size and shape), they could be associated to processing bodies (P-bodies, PBs), or even to stress granules (SGs). PBs have been described as cytoplasmic mRNPs aggregates mostly constituted by protein components involved in RNA degradation and turnover (DCP decapping enzymes, VARICOSE and XRN4) (Xu and Chua 2011). SGs are compositionally different from PBs, being composed by mRNAs that are stalled in the process of translation initiation producing a complex with translation initiation factors (eIFs), the 40S ribosomal subunit and PABPs (Balagopal and Parker 2009). These cytoplasmic structures normally assemble upon cellular homesostatic disturbance. SGs have been described by their temporal and spatial composition, size and activity of constituent factors (Weber *et al.* 2008). Once the present experimental approach corresponds to the over-expression of

HRR.1 and *HRR.2* transcripts and corresponding proteins, such a stressful condition could have resulted in the assembling of SGs.

The presence of GFP6-HRR.2 fusion in nuclear speckles suggests that HRR.2 could modulate of protein-protein interactions between splicing factors, mRNA decay processes, chromatin remodelation or even modifications in rRNA transcription and processing. Nuclear speckles, also known as interchromatin granule clusters (IGCs), are well described in mammalian cells and comprise irregularly shaped structures that vary in size (Fang *et al.* 2004; Biamonti and Vourc'h 2010). These sub-nuclear domains are located in the interchromatin regions of the nucleoplasm and were suggested to be sites for pre-mRNA splicing factors storage and/or reassembly. Splicing factors are recruited from these compartments to the sites of active transcription (Fang *et al.* 2004). The same subnuclear localisation was also described for some Arabidopsis SR proteins (SR1, SR30, SR33), whose sizes and number of speckles vary considerably with development and in different tissues (Ali and Reddy 2008b). Since these nuclear compartments are in constant interchanging, HRR.2 could be important for the regulatory nuclear roles. Hence, HRR.2 could interfere in the modulation of transcription (co-interaction with transcription machinery and chromatin reposition), post-transcription (expression of SR- and spliceosome-related transcripts) or post-translation (modifications in protein-protein interactions between SR and other spliceosome proteins).

The nucleoplasm localisation of HRR.1-GFP6 fusion suggests a possible nuclear role in transcript stabilisation during 5'-capping and 3' end-polyadenylation, such as the role played poly(A)-binding proteins (PABPs). The PABPs are highly conserved proteins between eukaryotes (Mangus *et al.* 2003). Single-celled eukaryotes only have a single PABP coding gene, whereas humans have five and *Arabidopsis* has eight. These proteins are mostly associated with mRNA maturation processes, where they play roles in synthesis of 3'poly(A) tail, mRNA export, translation initiation and termination and recycling of ribosomes. They are not only important for definition of poly(A) tail length during mRNA processing, but also their binding prevents the deadenylation, a step that normally results in mRNA decay (Mangus *et al.* 2003). HRR.1 could interact with PABPs during the nuclear mRNA maturation and cytoplasmic regulation of translation process. This hypothesis is in part corroborated with the predicted protein interaction and co-expression/regulation with PAB6 (Section 3.1.3).

The HRR.2-GFP6 fusion resulted in a weak GFP signal, which was detected in cytoplasmic granules and nucleus. In contrast, the HRR.1-GFP6 fusion presented a uniformly distributed GFP signal within the nucleus (Figure 3.37H and F, respectively). Altogether, the results suggest that HRR.1 and HRR.2 are both present in nucleus and cytoplasmic granules. However, they seem to have different subcellular accumulation in the nucleus. Comparing both GFP intensity signal, HRR.2

appears to be less accumulated in nucleus than HRR.1. This difference could be due to the presence of PABP-1234 functional domain in HRR.1 and not in HRR.2. The importance of presence/absence of specific functional domains were already reports for the U-rich binding proteins RBP47 and UBP1 (Weber *et al.* 2008). In this work, truncated proteins lacking the RNA binding domain or its prior domain differed in subcellular localisation as compared to native proteins. The functional PABP-1234 domain, present in HRR.1, could be important for transcript maintenance. Once the PABPs are normally associated to mRNA 3'UTRs, their C-terminal functional domains vastly interacts with other factors regulating several steps of RNA metabolism (polyadenylation, nuclear export, initiation and termination of translation, mRNA decay) (Mangus *et al.* 2003). In the absence of such functional domain in HRR.2 protein, essential protein interactions with other factors and stability of transcripts could be compromised. Predicting such conditions, both HRR.2 proteins and transcripts could be subjected to degradation processes.

To gain more insight into the expression and dynamic organisation of HRR.1 and HRR.2 proteins in the cell, their expression under the control of their endogenous native promoter was followed. Instead of the highly active constitutive *35S* promoter, new constructs harbouring the predicted *HRR* promoter (p*HRR*) for controlling the expression of N-terminal GFP6-HRR.1 and GFP6-HRR.2 fusions were obtained. *Nicotiana tabacum* cell suspensions of Bright-Yellow 2 line (BY2 cells) were used for the subcellular localisation analysis of expressed *pHRR:GFP6-HRR.1* and *pHRR:GFP6-HRR.2* transgenes (Figure 3.38). BY2 cells are easily transformed by Agrobacterium and present high sensitivity and capacity to withstand stress conditions, in comparation with Arabidopsis culture cells (Koroleva *et al.* 2009).

Under standard growth conditions (23ºC), both transformed BY2 cell lines displayed low levels of protein expression. When heat-stressed at 38ºC for 60 minutes, the GFP6 signal was much more intense. A stressful condition is thus necessary for the induction of these transgenes. In this plant model and after HS treatment, GFP6-HRR.1 fusion was detected in the nucleus (thick orange arrow) and cytoplasmic granules (light orange arrow), whereas GFP6-HRR.2 was only detected in cytoplasmic granules. These cytoplasmic granules are likely to be SGs or PBs. PBs have been described as spheric and uniform aggregates in size and shape, which increase in number and size in response to stress, while SGs are morphologically more heterogeneous and are assembled under stressful conditions (Weber *et al.* 2008). Accordingly, it appears that HRR.1 seems to be predominantly found in SGs, whereas HRR.2 seems to be found in PBs. However, the possibility of both proteins are present as in SGs as in PBs should not be excluded.

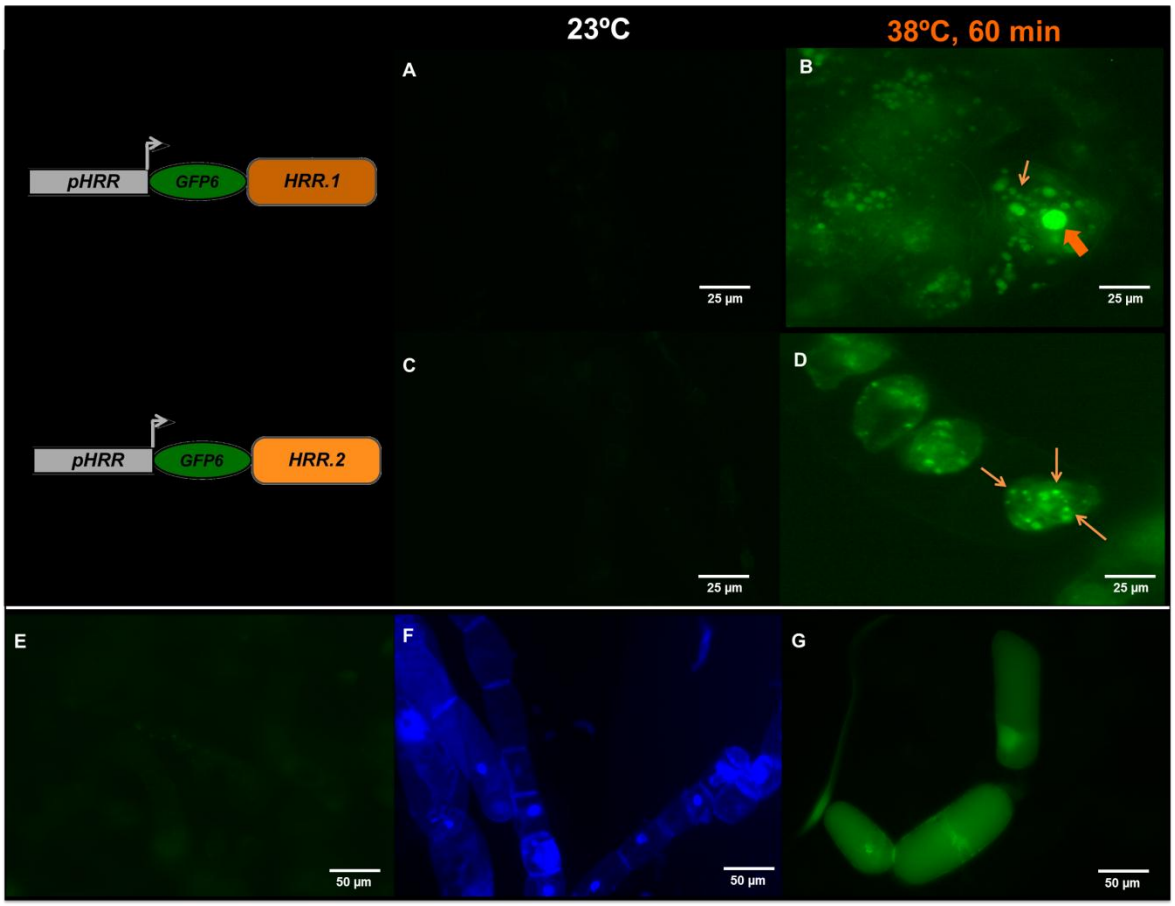


Figure 3.38 Subcellular localisation of native HRR.1 and HRR.2 fusions in tobacco (*Nicotiana tabacum*) cell suspension culture. Transformant tobacco Bright-Yellow2 (BY2) cells, harbouring the p*HRR::GFP6-HRR.1* and p*HRR::GFP6-HRR.2* transgenes were observed using fluorescence microscope. Transformant tobacco BY2 cells, under normal growth conditions, depicted basal expression (**A** and **C**, for HRR.1 and HRR.2, respectively). The intracellular localisation of HRR.1 and HRR.2 was detected after imposition of a HS treatment (HS, 38ºC for 60 minutes) (**B** and **D**, respectively). (**E**) Non- transformant tobacco BY2 cells were used as negative control. (**F**) DAPI fluorescence in non-transformed tobacco BY2 cells was used as a nuclear marker. (**G**) GFP fluorescence in transformant tobacco BY2 cells expressing p*35S::GFP* transgene was used as positive control. Orange arrows indicate cytoplasmic granules; thick orange arrow indicates the nucleus.

These results are somewhat similar to the corresponding ectopic expressions (Figure 3.37), except for GFP6-tagged HRR.2 protein. In this case, when natively expressed, HRR.2 fusion protein was only observed in cytoplasmic granules (SGs and/or PBs), not being observed its presence in nuclear speckles. Collectively, these results suggest that native expression of HRR proteins is HS-dependent. However, the different subcellular localisation for both proteins, under different promoter activities, suggests the importance of cellular physiology state for determination of HRR proteins targeting.

3.4.2 Functional dynamics of HRR under HS treatment

In an attempt to understand the dynamics under HS treatment, the transformed p*HRR*::*GFP6-HRR.1* BY2 cells were HS-stressed for different periods, ranging from 15 to 60 min (Figure 3.39). The GFP signal was followed to observe the SG assembly dynamics of cytoplasmic granules and association of HRR.1 with them.

After 15 min of HS treatment, HRR.1 fusion was still fairly detected. Hereafter, at 30 min of HS treatment, the HRR.1 fusion was detected in the nucleus, but also in the cytoplasm. After 60 min of HS treatment, the GFP signal was evident a broad quantity of GFP6-HRR.1 was observed in cytoplasmic granules.

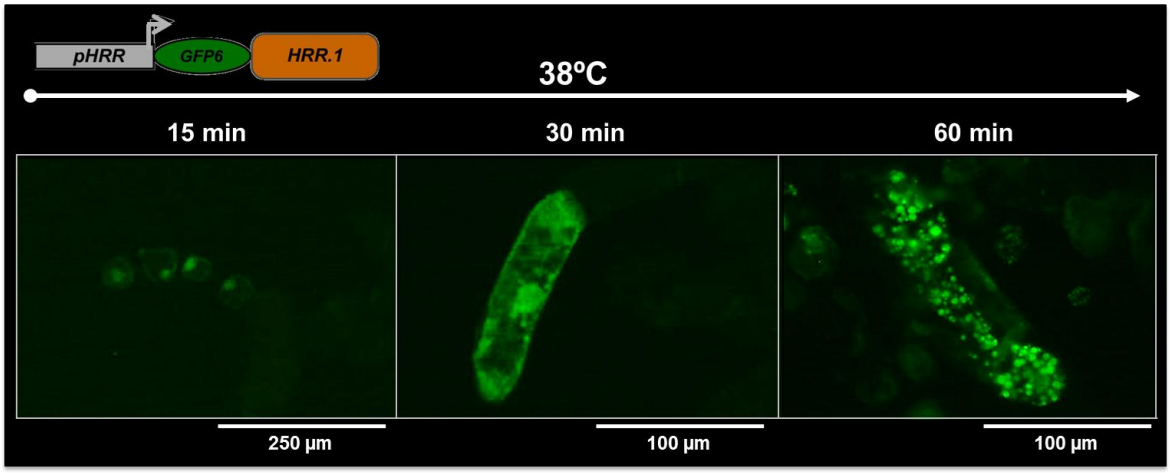


Figure 3.39 Intracellular dynamics of HRR.1 fusion protein in cytoplasmic aggregates after heat stress impositions. Transformant tobacco BY2 cells, harbouring the transgene *pHRR::GFP6-HRR.1*, were observed using a fluorescence microscope after imposition of 38ºC, for 15, 30 and 60 min (*Leica DM 5000 B*).

The temporal expression of HRR.1 fusion corresponds to the previous *HRR* expression analysis, where the highest *HRR* expression levels were reached one hour after HS treatment (Section 3.3.1). Although using different biological systems, both transcripts and protein reached highest levels after one hour of HS treatment (Figure 3.22 and 3.39). These results suggest that HRR is recruited in early stages of HS response. Even before, the GFP signal distribution during the first 30 min of HS treatment could be due to the association of HRR.1-containing granules to cytoplasmic microfilaments. In mammalian cells, the increasing of SG size was suggested to be facilitated by the transport of smaller SGs along microtubules with subsequent fusion of them (Nadezhdina *et al.* 2010). Hence, these results suggested that HRR.1 protein is expressed in early stages of HS imposition and could be progressively required for SG assembling. At this moment, it is impossible to

conclude if these cytoplasmic granules correspond to SGs or PBs. Following previous reported results, SGs are rapidly induced (15-30 min) in response to environmental stress and are heterogenous in size and shape (Anderson and Kedersha 2006). As the cytoplasmic foci reveal such morphological aspect and temporal formation, HRR.1 could be suggested to make part of SG composition. However, due to the interchangeable dynamic activity between SGs and PBs, HRR.1 could also be present in PBs.

Even before their description in mammalian cells, SGs were observed in cytoplasm of tomato cells subjected to HS treatment, being referred to heat stress granules (HSGs). At that time, the HSGs were described as cytoplasmic aggregates containing HSPs and untranslated housekeeping mRNPs, formed during long-term HS treatment (Nover *et al.* 1989). When first described in mammalian cells, the SGs presented a different composition, comprising stalled 48S preinitiation complexes (untranslated poly(A)+ mRNA bound to small ribosomal subunit), associated to core assembling RNA-binding proteins TIA-1 (T-cell internal antigen-1) and TIAR (TIA-related protein) (Kedersha *et al.* 1999; Kedersha *et al.* 2000). More recently, similar structures were characterised in plants. These granules contain 48S pre-initiation complexes associated to early translation factors (eIF4E) and many RNA-binding proteins (RBP47 and UBP1) (Weber *et al.* 2008). In contrast, PBs have a different composition, comprising a core enzymatic complex involved in mRNA decapping and 5'-3' exonucleotidic activity (DCP1, DCP2, DCP5, XRN4 and some components of NMD, as UPF1), as well as 3'-5' exosome-associated mRNA degradation (Souret *et al.* 2004; Potuschak *et al.* 2006; Xu *et al.* 2006; Goeres *et al.* 2007; Brogna *et al.* 2008). In addition to RNA decay, P-bodies also function in translation regulation (Xu and Chua 2011). Considering that UBP1 is one of strong candidates to interact with HRR.1 protein (section 3.1.3), it is likely that HRR.1 could mainly exert their functions in SGs.

3.4.3 Determination of the putative HRR role on the biogenesis of cytoplasmic aggregates

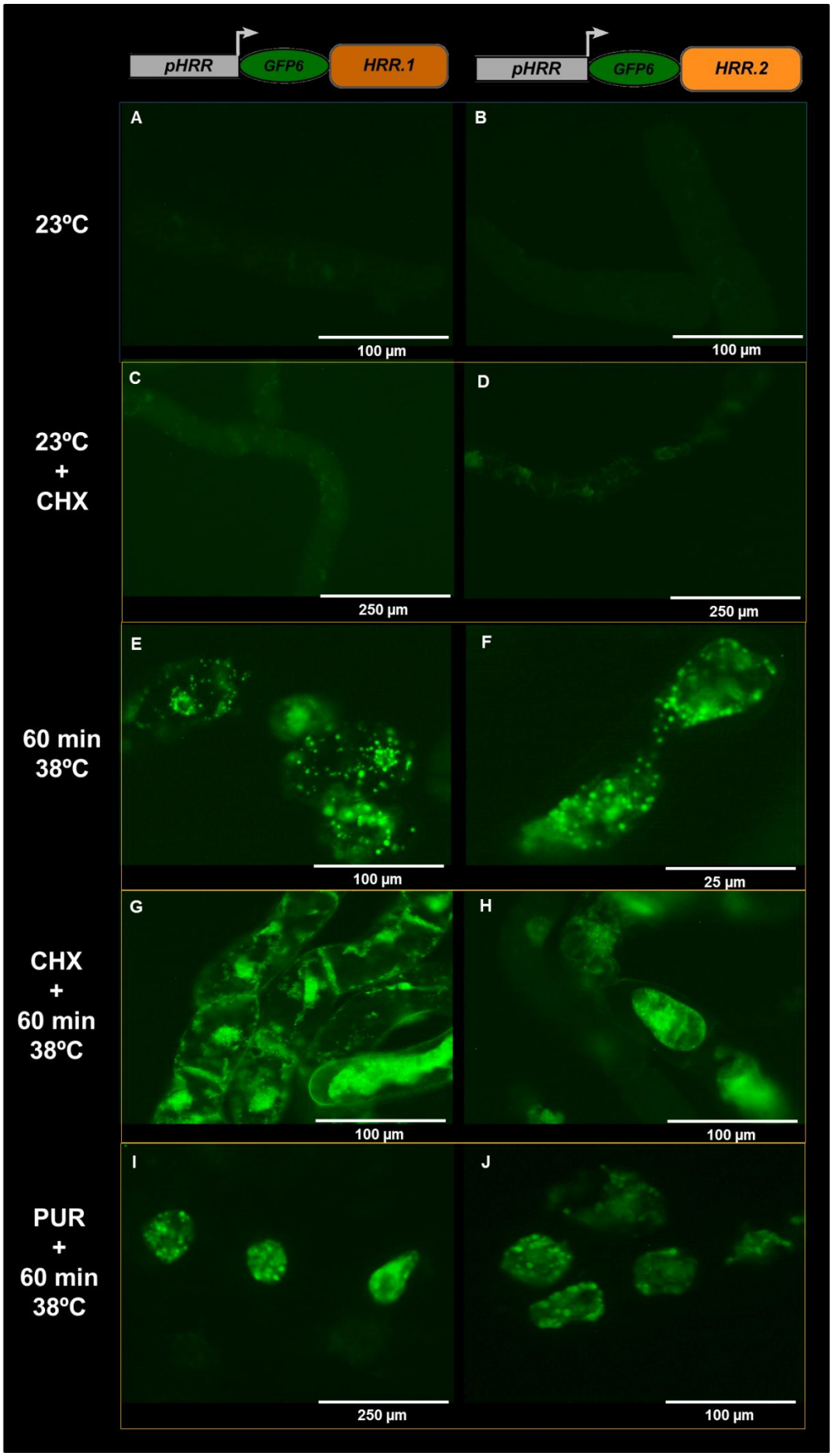
Taking into account that HRR.1 and HRR.2 could be targeted to SGs and/or PBs and the assembling of both structures depends on non-translated transcripts flux, the requirement of HRR proteins for assembling of such cytoplasmic RNA granules was investigated. For this, the assembly and putative dynamic exchange of components between these cytoplasmic mRNP-containing complexes was disrupted by chemical treatment. The application of cycloheximide (CHX) would inhibit the translation by blocking the mRNA release from polysomal complexes. Conversely, the application of another

translation inhibitor, puromycin (PUR), would have an inverse effect of CHX, by destabilising of polysomes and releasing the transcripts that were being translated. While CHX application promotes the ribosomes stalling on transcripts and inhibits the formation of stress granules, PUR application promotes the SG assembling. These results have been explained by the requirement of the inhibition of translation initiation, for SG formation (Anderson and Kedersha 2002; Weber *et al.* 2008).

When tobacco BY2 cells transformed with p*HRR::GFP6-HRR.1* and p*HRR::GFP6-HRR.2* transgenes were treated with CHX, under standard conditions (23ºC), the expression of HRR.1 and HRR.2 fusions slightly increased (Figure 3.40, C and D), comparing with non-treated BY2 cells (Figure 3.40, A and B). As previously observed (Figures 3.38 and 3.39), under HS treatment (38ºC, 60 min), a high number of fluorescent cytoplasmic aggregates was detected on p*HRR::GFP6-HRR.1* and p*HRR::GFP6-HRR.2* transgenic BY2 cells (Figure 3.40, E and F). However, when both transformant BY2 cells were treated with CHX and subsequently heat-stressed, a marked reduction in size and number of cytoplasmic granules was observed (Figure 3.40, G and H). The same transformant BY2 cells when treated with PUR and then heat-stressed, they displayed a re-assemblying of cytoplasmic granules (Figure 3.40, I and J)

In standard conditions, the exposition of transformant BY2 cells to CHX promoted a small accumulation of both tagged HRR proteins. In part, these results corroborate with bioinformatics data (BAR browser), which predict that HRR is margely up-regulated under CHX treatment (10 µM, by three hours). Besides cycloheximide treatment, HS condition also influences the HRR activity. Such as previously shown, a large amount of cytoplasmic granules was observed in both transformant BY2 cells (Figure 3.38). However, the spatial distribution of both HRR fusion proteins was different. GFP-HRR.1 fusion-containing granules were mostly found close to nuclear periphery and in cytoplasm, while GFP-HRR.2 fusion was more randomly dispersed in the cytoplasm. These results suggest that HRR proteins possess specific subcellular dynamics. The presence of HRR.1 fusion protein close to the nuclear membrane indicates that this protein could be recruited for mRNA nuclear export or translation initiation process. In mammalian models, the translation initiation is characterised as 'pionner round', consisting in the ribosomal scanning (searching by PTCs and EJC displacing) and remodelating of mRNP (Ishigaki *et al.* 2001; Chang *et al.* 2007).

The HRR.2 fusion, seems to be located in SGs, or PBs or in both, during HS treatment. The dynamic of SGs and PBs assembling is mostly dependent from mRNP homeostasis, not only in standard conditions as during stressful conditions. Hence, the exposition of transformant BY2 cells (GFP6-HRR.1 and GFP6-HRR.2) to translation inhibitors cycloheximide (CHX) and puromycin (PUR) allows to infer if HRR proteins are involved in formation of such RNA granules. The CHX treatment before HS imposition allows the evaluation of dynamic influx of stalled mRNPs from SGs to PBs,


pHRR
GFP6
HRR.1
pHRR
GFP6
HRR.2
23ºC
A
100 µm
B
100 µm
23ºC + CHX
C
250 µm
D
250 µm
60 min 38ºC
E
100 µm
F
25 µm
CHX + 60 min 38ºC
G
100 µm
H
100 µm
PUR + 60 min 38ºC
I
250 µm
J
100 µm

through the increase/decrease of PBs number and size. The formation of SGs and PBs has been described to be inhibited by application of CHX, in stresses cells (Weber *et al.* 2008). In addition, CHX-treated HeLa cells presented SGs dissociated into their constituents that were dissolved in the cytoplasm (Nadezhdina *et al.* 2010). Occurring the SG dissolution under CHX treatment, the mRNP flux between SGs and PBs is interrupted and RNA granules disappear. The decreased number and size of cytoplasmic aggregates after CHX treatment and subsequent HS imposition indicates that HRR.1 and HRR.2 could be involved in SG and PB assembling. As a result, the composition of PBs would change, reducing their size and number. Conversely, the treatment with puromycin promoted the cytoplasmic aggregates assembling, after the HS treatment. This translation inhibitor is an aminoacyl tRNA analogue that destabilizes polysomes by promoting premature termination (Kedersha *et al.* 2000). Altogether, HRR proteins are suggested to be involved in dynamic flux of mRNPs between SG and/or PBs cytoplasmic RNA granules.

As a conclusion, the results suggest that the products of *HRR* alternative splicing, HRR.1 and HRR.2 proteins, could follow different subcellular pathways, during the thermotolerance responses upon HS conditions. Once translated, their targeting and intracellular accumulation appears to be somewhat different. In early responses to HS, HRR.1 could promote the SG assembly, participating in recruitment of stalled and housekeeping mRNPs, possibly through the protein-protein interactions with other RNA-binding proteins. The untranslated mRNPs are then screened for (1) storage, (2) reintegration into translation program/process or (3) moved to PBs, where are expected to be degraded. All these tasks can only be afforded by a dynamic exchange of components between SGs and PBs, in which HRR.1 is likely to be involved. Even though HRR.2 could be early integrated into SGs, most of its corresponding GFP6 signal was observed in cytoplasmic granules similar to PBs, which were smaller than SGs. HRR.2 could play a regulatory function during mRNA decay and transcriptional regulation, upon HS conditions. The modified HRR.2 binding motif (in RRM domain) could be sufficient for altering the RNA and protein interaction activities. A specific set of transcripts could then be drived for degradation, including their transcripts. In PBs, the transcripts can be degraded through 5'-3' degradation pathway (NMD), or HRR.2 transcripts could be also degraded by exosome (3'-5' degradation), generating small RNAs. Ultimately, these molecules might be recruited to the nucleus, where could influence the transcriptional activity.

(Left page) Figure 3.40 Intracellular dynamics of HRR.1 and HRR.2 fusions under HS conditions and chemical treatment (CHX and PUR). Transformant BY2 cells harbouring the p*HRR::GFP-HRR.1* and p*HRR::GFP6-HRR.2* transgenes were observed using a fluorescence microscope. Cells were observed under standard conditions (**A** and **B**, respectively), after being treated with cycloheximide (CHX, 100 µg.ml^{-1}; **C** and **D**, respectively) or upon HS treatment (38ºC, 60 min; **E** and **F**, respectively). The transformant BY2 cells harbouring *pHRR::GFP6-HRR.1* (**G**, **I**) and *pHRR::GFP6-HRR.2* transgenes (**H**, **J**) were treated with CHX (100 µg.ml^{-1}) (**G** and **H**) or treated with puromycin (PUR, 10 µg.ml-1) prior of HS treatment (**I** and **J**), 60 min prior to HS imposition.

4. FINAL REMARKS AND FUTURE PERSPECTIVES

4.1 Final Remarks

During evolution, plants have developed several survival strategies to cope with environmental cues. Particularly, under high field temperatures, plant adaptation is achieved through a broad range of morphological, physiological and molecular responses. Plant responses to HS are mainly determined by key molecular reorganisations, affecting different levels of gene regulation. Post-transcriptional regulation under HS conditions is mostly carried out by RNA-binding proteins. In recent years, some RNA-binding proteins (most RRM-containing proteins) have been described, but few studies have been achieved in attempt to understand their involvement in HS-associated transcriptome regulation. The fundamental knowledge obtained from these studies will contribute for the improvement of crop plants of great agronomic and economical interest. The work presented in this thesis allowed to uncover the molecular roles of an Arabidopsis RNA-binding protein that is highly and specifically expressed under HS conditions. This will allow to get new insights on plant adaptive post-transcriptional mechanisms during HS imposition.

The availability of Arabidopsis transcriptomic data, provided by ATH1 Gene Chip experiments, allowed the previous selection of several heat-responsive genes. Among them, *HRR* (*Heat-Responsive RNA-binding protein*) gene encodes a putative RNA-binding protein that could be involved in binding transcripts and other RNA-binding proteins during heat stress, thus representing a potential determinant gene for thermotolerance. The principal objective of this thesis was to functionally characterise the HRR protein, using bioinformatic, phenotypic and molecular approaches.

HRR is a RNA-binding protein that contains a N-terminal RRM domain and a C-terminal PABP-1234 functional domain. The presence of this functional domain suggests putative roles on transcript stabilisation, transport of mature mRNAs and/or translation initiation. The phylogenetic alignment of HRR with different Arabidopsis RRM-containing proteins (belonging to different functional groups) and potential metazoan HRR orthologues revealed that HRR is closest to GR-RBPs and oligouridylate-binding proteins. Although structurally different at C-terminus, the phylogenetic relationship of HRR with AtGR-RBPs may indicate related functions. The AtGR-RBPs are actually the RRM-containing proteins that display well-characterised stress-related functions (Kwak *et al.* 2005; Kim and Kang 2006; Kim *et al.* 2007; Kim *et al.* 2008b). The majority of AtGR-RBPs already investigated appear to be involved in responses to different stress conditions, namely cold, salinity and drought. AtGR-RBP2, AtGR-RBP7 and AtRZ-1a (which possesses a RNA helicase activity) have been described as being crucial in promoting seed germination and seedling growth under cold stress and their over-expression confers freezing tolerance (Kim *et al.* 2007; Kim *et al.* 2008b; Kim *et al.* 2010). GR-RBPs have been

indicated as important regulators in pre-mRNA processing and/or stability of mRNAs. They promote the better RNA conformation for RNA transport and translation processes enhancement under those conditions (Zdravko J 2009). Although the different physiological and molecular features between HS and other abiotic stress conditions, HRR could play similar functions under HS conditions. In addition to GR-RBPs, the phylogenetic closeness of HRR with oligouridylate-binding proteins (UBA, UBP and RBPs) could also reveal mRNA stability functions for HRR. U-rich binding proteins were early characterised as being involved in maturation of plant pre-mRNAs, promoting the stabilisation of transcripts by recognition of AU-rich sequences present in 3'UTR (Lambermon *et al.* 2000; Lambermon *et al.* 2002). Indeed, they appear to be involved in recognition and stimulation of intron splicing (Lorkovic *et al.* 2000). More recently, these proteins have been implicated in responses to environmental cues. Through immunofluorescence studies, Weber *et al.* (2008) reported that, under HS conditions, RBP47 and UBP1 proteins are involved in cytoplasmic stress granules formation associated with untranslated poly(A)+ mRNAs. Thus, HRR could be suggested to play a role in the stability of HS-induced transcripts, most likely through the interaction with other RNA-binding proteins. The presence of stress-inducible *cis*-elements in *HRR* promoter (RAV/AP2, MYB and bZIP) and predicted HRR co-interaction with oligouridylate-binding proteins, spliceosome factors, RanGAP and PABP6 suggests that HRR could interfere in several steps of RNA metabolism, regulating HS-induced transcripts. Their putative role in different stages of RNA metabolism could occur due to the interaction with other RNA-binding proteins, probably playing crucial regulatory functions in different mRNP complexes.

Phenotypic analysis of *hrr* knockout and HRR over-expression mutants showed that HRR is strongly involved in seed thermotolerance responses. The phenotype observed in early stages of HS treatment (up to 60 min) suggests that HRR could play a role in the transition to a HS-specific response. Accordingly, the highest *HRR* expression levels were observed after 60 min of HS treatment. The involvement of HRR in the early HS responses is also corroborated by the down-regulation of *HRR* in the HSFA1 quadruple mutant (QK) (Liu *et al.* 2011). Considering that HSFA1 TFs are early master regulators in HS response, it is possible that *HRR* could be indirectly up-regulated by these factors, being requested for post-transcriptional regulation of downstream genes. HRR was also suggested to be involved in the stability of *HSFA2* transcripts, during HS treatment and recovery periods. HSFA2 has been described as a pivotal regulator factor in the expression of an extensive broad range of HS-related genes, promoting the acquired thermotolerance response (Schramm *et al.* 2006). As the tight and coordinated regulation of *HSFA2* expression levels is of extreme importance in response to a specific stressful input, the HRR function in the *HSFA2* transcripts stabilisation would be possible. Besides the germination impairment of *hrr* mutant seeds

detected after subjecting seeds to a heat treatment, a germination deficiency was also observed under salt stress and combination of heat and salt stress conditions. These results suggest that HRR could be involved in responses to the ionic imbalance imposed by salt stress. The possible cumulative effect between heat- and salt-induced stress responses may indicate that HS- and salt-signalling pathways play in parallel and HRR could regulate or protect a common set of stress-responsive (HSPs, LEA, antioxidant and osmolyte synthetic enzymes, calcium sensors, kinases and Na+/H+transporters) transcripts. Hence, HRR could be essential for the stability of such transcripts, whose products are important for cellular integrity under extreme conditions. The HRR involvement in salt responses and combination of stresses (HS and salt) is corroborated by the possibility of *HRR* be induced by TFs, which are up-regulated and act during early imposition of HS and salt stress conditions. DREB2A and DREB2B (ERF/AP2 transcription factors) are highly induced by salt/drought and high temperature responses (Sakuma *et al.* 2006b). These factors are up-regulated by HSFA1s and are involved in transcriptional regulation of later HS-induced *HSFA2* and *HSFA3* genes (Schramm *et al.* 2008). Considering that *HRR* could be indirectly induced by HSFA1, it is likely that DREB2A/2B factors up-regulate *HRR* expression, both under HS and salt conditions. Ultimately, HRR could promote the stability of HS- and/or salt-related transcripts. However, it should be considered that HS and salt-responsive transcriptomes might be different from the transcriptome associated to the combination of heat and salt stresses. For instance, the HRR function in all these experimental situations could be slightly different.

Hormonal sensitivity germination assays revealed that HRR could act as a positive regulator in ABA metabolism and signalling. In contrast, HRR appears to exert negative regulatory effects in GA pathway components. The lower ABA sensibility displayed by *hrr* mutant seeds in comparison to wild-type *Ler* could be explained by the accumulation and enhanced stability of ABA-related transcript levels, promoted in part by HRR (Figure 4.1). *HRR* is expressed during the later stages of seed maturation and germination. As *ABI5* levels were impaired in *hrr* mutant during later stage of seed maturation and at germination, HRR appears to be important for the regulation of *ABI5* transcript levels. This bZIP transcription factor is known to act as a master regulator in ABA signalling pathways, regulating the transcription of many ABA-related downstream genes, such *Em* genes that code for LEA proteins (Carles *et al.* 2002). Simultaneously, HRR could also regulate the *HSFA9* transcripts, whose product is responsible for the induction of *HSP* genes, namely *HSP101* (Figure 4.1). Indeed, *hrr* mutant seeds display lower levels of *HSP101* transcripts during germination compared to wild-type *Ler* seeds. The accumulation of LEA proteins, HSP101 and other protective proteins during seed maturation and early events of germination is crucial for proteins integrity and determine the seed longevity and germination ability.

Equally important for seed germination is the regulation of the positive feedback mechanism established during ABA biosynthesis. This regulation is extremely important and mainly occurs during seed germination, where the increasing GA levels counteract the early raising of ABA levels (Figure 4.1). At this stage, *hrr* mutant displayed lower levels of ABA metabolism (*ABA1* and *NCED9*) and GA signalling (*SPY*) transcripts than wild-type *Ler*, suggesting a putative role of HRR on the stability and possibly the turnover rate of these transcripts. This would ensure the tight control of ABA-related enzymes, including the rate-limiting ABA biosynthetic (NCED9) enzyme levels. Under HS conditions, the accumulation of *ABI* and ABA biosynthesis transcripts in *hrr* mutant seeds reveals the importance of HRR in the control of seed thermoinhibition. This defense mechanism should be tightly regulated, once it could dictate the seed germination ability at recovery period.

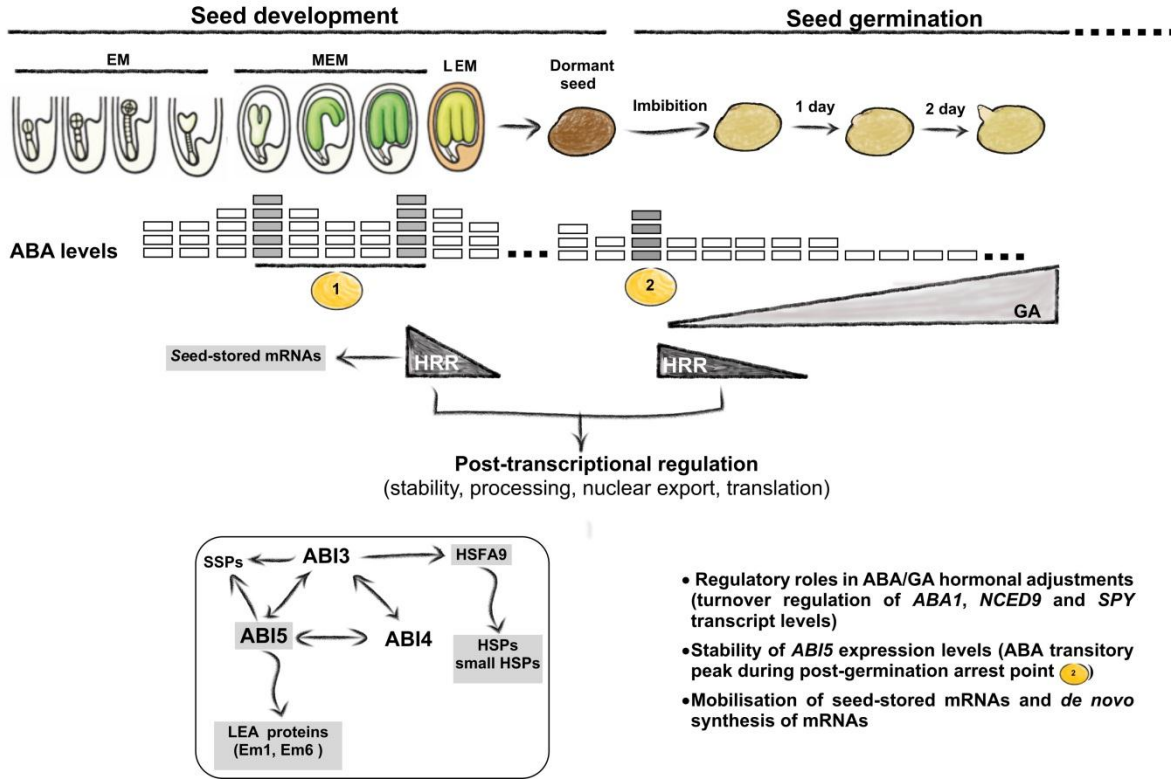


Figure 4.1 Proposed model for HRR expression and its possible role during seed development and germination. During seed development, two ABA peaks occur (1). The first occurs after the cell division arrest and promotes the synthesis of storage-related mRNAs and proteins. The second ABA peak occurs during mid-maturation stage and may stimulate the synthesis of LEA proteins and prepare the embryo for desiccation. Based on the results from gene expression analysis, *HRR* appears to be up-regulated during the second ABA peak, being possibly involved in the metabolism of *ABI5, HSFA9, HSP101* and *LEA* protein transcripts (inside a grey box). During early stages of seed germination (seed imbibition), *HRR* is again up-regulated and coincident with a transitory increase of ABA levels (2). Under tight regulation of ABA/GA homeostasis, HRR could be responsible for the stability and turnover regulation of ABA metabolism (*ABA1, NCED9*) and signalling (*ABI5*), as well as SPY (GA negative regulator) transcripts. Indeed, HRR could be involved in regulation of seed-stored and new synthesised mRNAs. Seed development comprises the following stages: EM (Embryo Morphogenesis), MEM (Mid Embryo Maturation) and LEM (Late Embryo Maturation).

Altogether, HRR is proposed as a positive regulator component in ABA metabolism and signalling pathways, possibly regulating key transcript levels during the positive feedback loop in ABA biosynthesis during germination. This RNA-binding protein could directly or indirectly promote the stability of transcript levels corresponding to ABA biosynthesis enzymes (*ABA1*, *NCED9*) and ABA signalling proteins (*ABI5*), as well as stress-related proteins (*LEA* proteins and *HSPs*). The absence of HRR in *hrr* mutant during seed maturation may be in part responsible for the lack of post-transcriptional regulation of crucial transcripts, whose products would be important to regulate the transition phases from seed dormancy to germination.

In contrast to ABA, HRR seems to exert negative effects in the post-transcriptional regulation of GA metabolism. The hypersensitivity of *hrr* mutant under exogenous GA treatment could be in part correlated with the low levels of *SPY* transcripts during seed germination process in comparison to wild-type *Ler* transcript levels. Together with DELLA proteins, this GA negative regulator is responsible for the negative control of GA biosynthesis (Olszewski *et al.* 2002). The involvement of RNA-binding proteins regulating transcripts of GA-related genes, either directly or indirectly, have never been reported. Nevertheless, as GA homeostasis in different developmental stages is controlled by a negative feedback mechanism, some RNA-binding proteins are likely to regulate the transcript levels of GA metabolism and signalling genes.

Globally, the results indicate that the HRR functional role could be influenced by the plant hormonal balance. HRR could play important roles during the transition of developmental stages, mostly from dormant to germinated seed. During this transition, a tight control of gene expression and a fine tune balance between the ABA and GA hormones would be crucial for further plant development. Other RNA-binding proteins are also regulated by ABA levels. The AKIP1 protein (a *Vicia faba* RBP that interacts with kinase AAPK) binds with high affinity to dehydrin transcripts under stress conditions (Li *et al.* 2002). In addition, AKIP1 is partitioned to nuclear speckles under ABA treatment. The same phenomenon was observed with its closest Arabidopsis homologue UBA2a, which is an interacting partner of hnRNP-like UBP1 protein (Lambermon *et al.* 2000; Lambermon *et al.* 2002; Riera *et al.* 2006). The transgenic plants expressing the constitutively nuclear UBA2a-GFP also formed nuclear speckles after ABA treatment. Since the nuclear speckles of mammalian systems are implicated in storage of splicing components, pre-spliceosome assembling, mRNA stability and active RNA processing, AKIP1 and UBA2a proteins are likely to be involved in similar molecular mechanisms (Melcak *et al.* 2001). As UBP1 is functionally complexed with UBA1 and UBA2 proteins (Lambermon *et al.* 2002) and is predicted to co-interact with HRR, it is possible that HRR also takes part of such oligouridylate-binding protein complex during some steps of RNA metabolism involving ABA-related transcripts.

After HS imposition in seedlings, *HRR* expression was subjected to an alternative splicing process, originating the canonical *HRR.1* and the intron-retained *HRR.2* transcripts. By possessing an in-frame PTC, the *HRR.2* alternative transcripts are good targets for degradation (mostly through NMD mechanism). However, the occurrence of alternative splicing does not always occur. For instance, in non-stressed and HS-treated imbibed seeds, only *HRR.1* transcripts were expressed. This could suggest that the role of each HRR protein could not be exactly the same at different plant development stages and under different environmental conditions. *In vitro* analysis of HRR fusion proteins revealed that both proteins possess different functional dynamics and could follow different subcellular pathways, under HS treatment. HRR.1 appears to be located in the nucleus, nuclear periphery and cytoplasmic granules, while HRR.2 was mostly associated to cytoplasmic granules. Cytoplasmic granules has been proposed to be SGs and/or PBs. SGs function as triage cytoplasmic compartments that accumulate untranslated mRNPs, while PBs are associated with mRNA turnover and translation regulation. Due to their heterogeneity level, HRR.1 appears to be more associated to SGs, while HRR.2 seems to be related to PBs. However, as the PB activity depends from SG biogenesis and activity, HRR.1 is possibly interchanged between SGs and PBs.

The different subcellular localisations of both HRR proteins could be related with the presence/absence of the functional domain PABP-1234 in HRR.1 and HRR.2, respectively. The results obtained in this work could suggest a model for the cellular functional role of each HRR protein, under HS conditions (Figure 4.2). Due to the presence of PABP-1234 domain, HRR.1 could be involved in the nuclear mechanisms of mRNA processing, stability and mRNA export of housekeeping and HS-induced transcripts. The presence of HRR.1 fusion protein close to the nuclear membrane also suggests a possible role on mRNA nuclear export or translation initiation process. In addition, through protein-protein interactions with other RNA-binding proteins present in SGs, HRR.1 could participate in different roles of SGs, such as (i) regulation of stalled polyribosomes and non-translated housekeeping mRNPs, (ii) triage of mRNPs, keeping them in SGs, re-introducing them in the translation process or driving them for PBs for degradation, and (iii) regulation of dynamic interchange of RNA-binding proteins and other related factors between SGs and PBs. Concerning the *HRR.2* transcript, the presence of a PTC predicts its instability and premature translation termination. Indeed, the *HRR.2* transcript levels are determined by the transcription rate of *HRR* gene. Due to their higher instability, *HRR.2* transcripts are less trapped in polysomes upon CHX treatment, suggesting that at least part of HRR transcripts are retained and degraded in nucleus through a NMD mechanism.

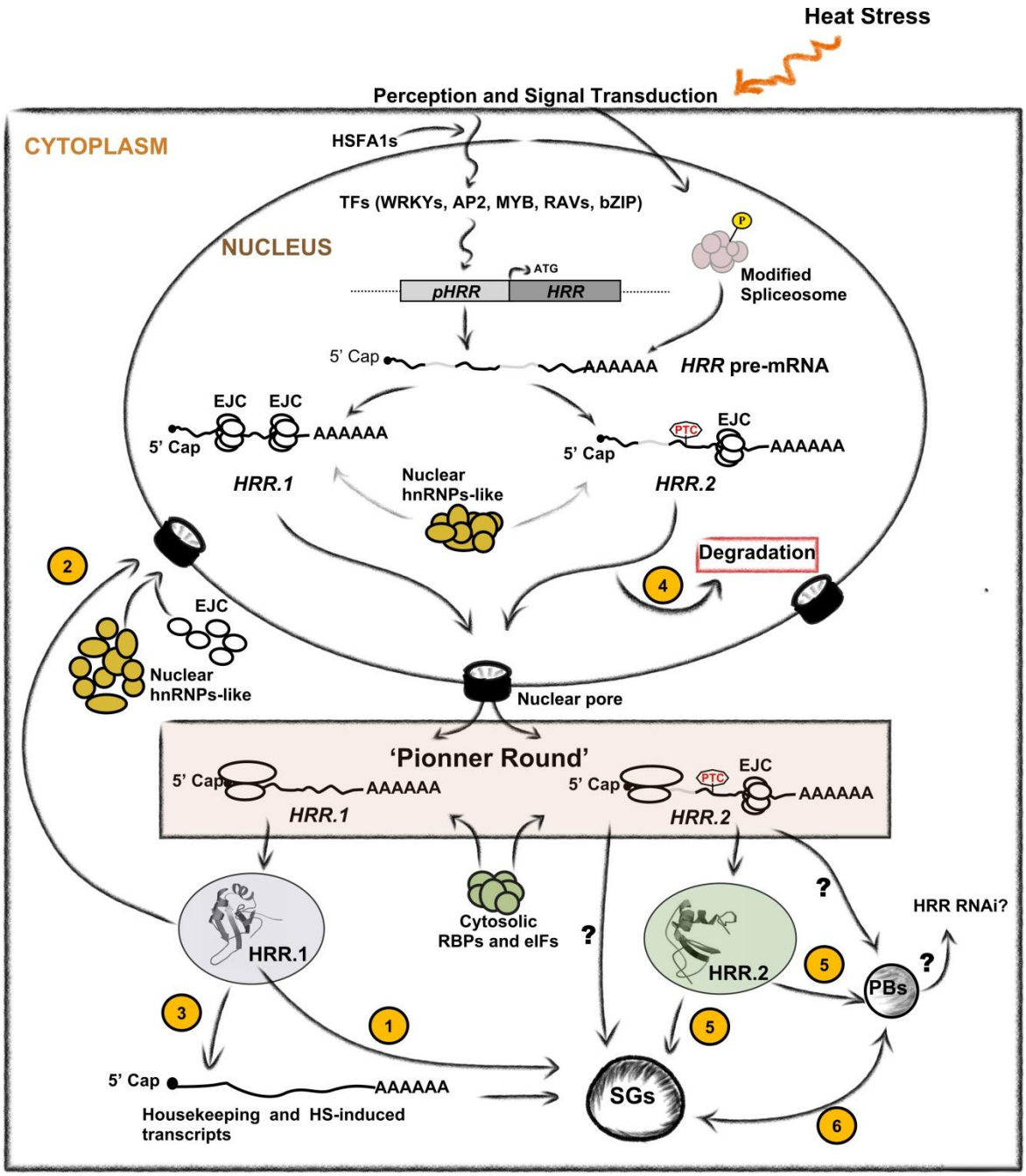


Figure 4.2 Proposed model for the functional roles of HRR.1 and HRR.2 under HS conditions. After HS perception, HSFA1s promote the induction of HS-responsive TF genes, which could induce the *HRR* expression. In turn, the spliceosome machinery is susceptible to successive cycles of phosphorylation/dephosphorylation, altering its splicing activity over *HRR* transcripts. From this process, two alternative transcripts are produced: *HRR.1* and *HRR.2*. During nuclear export, *HRR.1* and *HRR.2* transcripts could follow different pathways, depending on the "pioneer round" translation process. Once translated, HRR.1 could participate in the biogenesis and activity of SGs (1), possibly being responsible by stabilisation and translocation of housekeeping and HS-induced transcripts (3) HRR could also be recruited to the nucleus or be integrated in the complex involved in mRNA quality control performed during mRNA nuclear export (2). Due to the presence of an in-frame PTC, *HRR.2* transcripts, could still be retained in the nucleus, being further degraded (4). Those *HRR.2* transcripts that skip the mRNA scanning control could be translated in the cytoplasm producing the HRR.2 protein. This truncated protein could interact and interfere with PBs and SGs activity (5). Both HRR.1 and HRR.2 could interchange between SGs and PBs (6). EJC, exon junction complex; hnRNPs, heterogenous ribonucleoproteins; RBPs, RNA-binding proteins; eIFs, eukaryotic initiation translation.

The biogenesis of cytoplasmic RNA granules is not strictly connected with perception of stressful conditions. Contrary to SGs, PBs not only are functionally requested during stressful situations, but also appear to be present during the plant development transition phases where they tightly regulate the gene expression. Recently, PB components (DCP1, DCP2, DCP5, VARICOSE and XRN4) from Arabidopsis were reported to be required for post-embryonic development (Xu *et al.* 2006; Xu and Chua 2011). As HRR could be involved in the regulation of specific transcripts during the later stages of seed development and germination, it is likely that HRR could interact with PB components during these transition phases. In this case, HRR could be integrated with PB components, promoting the triage selection of transcripts for storage or degradation. During seed maturation process until desiccation, many mRNAs are known to be stored, including the *SSP* mRNAs which have been reported as PB substrates. Indeed, *SSP* transcripts were highly accumulated during early germination in the decapping mutants *dcp1-1*, *dcp2-1* and *dcp5-1* (Xu *et al.* 2006). Possibly, HRR could interfere in the RNP metabolism, during transition from dormant seed to germinating seed, promoting the stability of transcripts or cooperating in the decapping and degradation of specific sets of transcripts.

The existence of a flux of HRR proteins between SGs and HSGs (heat stress granule) components should not be excluded. In previous plant thermotolerance studies, HSP101 was reported as possessing RNA-binding activity (Hong and Vierling 2001). Taking this into account, for longer periods of HS treatment, a specific set of HSPs and sHSPs could aggregate with specific HS-induced transcripts, enhancing their translational activity under stressful conditions. Once HRR appears to be involved in stability regulation of HS-responsive transcripts (*HSFA2* and *HSPs*), HSGs could be assembled in which HRR would promote the stability of *HSP* transcripts.

The sequence of possible events involving the predicted HRR proteins could culminate in a profound remodelation of transcriptome in response to HS imposition. The HRR intervention in transcriptome modulation could be promoted by the selection and stability of transcripts that will proceed to pre-mRNA processing, mRNA transport, translation initiation and decay mechanisms. Being HS an environmental factor with a tremendous impact in plant gene regulation, the homeostasis re-establishment is in part powered by the cellular ability in removing aberrant transcripts and truncated proteins through the most diverse decay and turnover cellular mechanisms. The failure in regulation of such mechanisms could culminate in high level of cellular toxicity, thus compromising the cellular viability under extreme conditions.

4.2 Future perspectives

The putative HRR function in transcripts stability and regulation suggests an important biological role, both under HS conditions and during seed development and germination. Further experiments will help to fill the gap between described molecular associations and the way they can affect the plant homeostasis. Some results from this work could be confirmed to give more information about HRR.

- The phenotypic analysis of HRR over-expression lines: salt stress, exogenous ABA and GA should be reproduced and the quantitative expression analysis of HRR and specific-responsive genes should be performed. This phenotypic analysis could include different experimental approaches, by differential combination of HS and salt stresses (HS treatment prior or after salt stress imposition). This analysis will allow to better define the involvement of HRR in abiotic stress- and phytohormonal-responsiveness pathways.
- The *HRR.2* transcript instability should be assessed using qPCR approaches. After an initial *HRR* induction by HS, the alternative *HRR* transcripts decay should be followed in the presence of chemical inhibitors and for extended HS treatment periods. This analysis will allow to determine whether *HRR.2* transcripts are degraded through nuclear RNA degradation mechanisms or degraded via NMD mechanisms in cytosol.

In an attempt to complement the developed tools for the functional characterisation of HRR, other strategies could be followed, such as:

- Transcriptomic analysis of *hrr* and/or HRR over-expression mutant lines, under HS and during seed development and germination. The identification of down- or up-regulated genes could give new hints about the targets of HRR function. From this knowledge, new mutants could be obtained to understand which the potential targets of HRR are, thus describing a possible regulatory pathway.
- RNA-protein (RNA immunoprecipitation) and protein-protein (yeast two-hybrid) interaction studies, both under HS conditions and during seed maturation and germination. From RNA immunoprecipitation analyses, it will be possible to know if HRR is involved in the direct binding to RNA molecules and which type of RNAs are bound. Following this strategy, the identification of immunoprecipitated RNAs could be achieved. From yeast two-hybrid interaction studies, the most probable HRR interacting partners could be predicted. This

would be useful for predicting the most probable cellular compartment in which HRR is integrated.

- *In vitro* co-localisation of HRR protein fusions with oligouridylate-binding proteins (UBPs, UBAs and RBPs), as well as RNA degradation-related proteins (DCP1 and DCP2, XRN4) and initiation translation factors (eIF4E). This analysis should be performed in wild-type *Ler* and *hrr* mutant Arabidopsis protoplasts, under standard and HS conditions. Furthermore, the subcellular localization of HRR protein fusions in HS-treated seedlings of RNA degradation mutants (*upf*, *dcp* and *xrn4* mutants) could also be performed. These studies will define in which cytoplasmic granules HRR proteins are (SG, PB and/or HSG) and determine their functional roles in SG and PB biogenesis and activities, during HS imposition.

Performing these proposed tasks, a better acknowledge about the functional roles of HRR under HS conditions and during seed development/germination could be obtained.

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6. ANNEXES

ANNEX I: RRM-CONTAINING PROTEINS and HRR ORTHOLOGUES

Protein sequences of *A. thaliana* RRM-containing proteins and HRR orthologue used in this work were obtained from NCBI (*UniGene* database) through the respective accession codes depicted in table A.

Table A. Representative *A. thaliana* functional groups of RRM-containing proteins and HRR orthologues used in this work and their accession codes for nucleotide and protein sequences.

Functional group	Name	AT code/ Nucleotide ID (NCBI)	Protein ID (NCBI)
Poly(A)-binding proteins (PABPs)	AtPABP1/PAB 6	At3g16380	NP_188259
	AtPABP2	At4g34110	NP_195137
	AtPABP2a	At5g10350	NP_196597
	AtPABP3	At1g22760	NP_173690
	PABP4	At2g23350	NP_179916
	PABP5	At1g71770	NP_177322
	PABP6/PABP 7	At2g36660	NP_181204
	PABP8/PAB7	At1g49760	BAB11475
	PABP9	At1g45100	NP_175125
Serine/Arginine-rich proteins (SR proteins)	AtSCL28	At5g18810	NP_197382
	AtSCL30	At3g13570	NP_187966.1
	AtSCL30a	At3g13570	NP_187966.2
	AtSCL33 (SR33)	At1g55310	NP_564685
	atSC35	At5g64200	NP_201225
	SR45	At1g16610	NP_173107
Small nuclear ribonucleoproteins (snRNPs)	AtU1A	At2g47580	NP_182280
	At70K	At3g50670	NP_190636
	AtU2B	At2g36260	NP_180585
	AtU2AF35a	At1g27650	NP_199096
	AtU2AF35b	At5g42820	NP_174086
	AtU2AF35-like	At1g10320	NP_172503
	AtU2AF65a	At4g36690	NP_195387
	AtU2AF65b	At1g60900	NP_176287

Oligourydilate-binding proteins	**RBP45a**	At5g54900	NP_568815
	RBP45b	At1g11650	NP_172630
	RBP45c	At4g27000	NP_567764
	RBP47a	At1g49600	NP_175383
	RBP47b	At3g19130	NP_188544.1
	RBP47c	At1g47490	NP_175180
	RBP47c'	At1g47500	NP_175181
	UBP1a	At1g54080	NP_175810
	UBP1b	At1g17370	NP_564018
	UBP1c	At3g14100	NP_188026
	UBA2a	At3g56860	NP_567042
	UBA2b	At2g41060	NP_181639
	UBA2c	At3g15010	NP_188119
	UBA1a	At2g22090	NP_565525
	UBA1b	At2g22100	NP_565526
	UBA1c	At2g19380	NP_565450
Glycine-rich RNA-binding proteins (GR-RBPs)	**GR-RBP1**	At2g16260	NP_179222
	GR-RBP2	At4g13850	NP_193121
	GR-RBP3	At5g61030	NP_200911
	GR-RBP4	At3g23830	NP_189025
	GR-RBP5	At1g74230	NP_177563
	GR-RBP6	At1g18630	NP_173298
	GR-RBP7	At2g21660	NP_179760
	GR-RBP8	At4g39260	NP_195637
HRR orthologues	**AtHRR**	At5g53680	NP_200179.1
	SUP-12 (*C. elegans*)	NM_001136466.1	NP_001129938
	XSEB4 (*X. laevis*)	NM_001089144.1	NP_001082613
	RBM38 (*M. musculus*)	NM_019547.2	NP_062420
	RBM38b isoform (*H. sapiens*)	NM_183425.1	NP_906270

ANNEX II: STANDARD PROTOCOLS

1. Seed sterilisation and germination

Arabidopsis seeds were stratified (4ºC, 2 days), in the dark. Seed sterilisation was performed as described in (Weigel and Glazebrook 2002), with some modifications. The stratified seeds were washed in 1 ml of ethanol 80% (v/v), for 5 min. Ethanol was replaced by 1 ml of sterilisation solution [15% (v/v) commercial bleach with 3.5% (w/v) effective chloride; 0.2% (w/v) SDS] and seeds were incubated for 10 min, with occasional vortexing. Seeds were then washed with 1 ml of sterile distilled water for three times. Between water washings, seeds were centrifuged at 13,000 *g* for 20 sec, for the appropriate removing of solutions. An additional water washing was performed and seeds were further incubated for 5-10 min, to remove the hypochlorite remains. After water discarding, seeds were resuspended in sterile 0.25% (w/v) agarose solution and sown onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962), containing 1x basal salt mixture (Duchefa), 1.5% (w/v) sucrose, 0.5 $g.L^{-1}$ MES (pH 5.7) and solidified with agar [0.8% (w/v) for horizontal growth or 1.2% (w/v) for vertical growth]. The plates were sealed with parafilm to prevent desiccation and placed in the growth room, under a long photoperiod (16 h light/ 8 h dark) with 80 $\mu E.m^{-2}.s^{-1}$ light intensity, at 23ºC. All procedures were performed under aseptic conditions in a horizontal laminar flow chamber (OSN).

2. Cultivation of plants on soil for bulk seed production

Seedlings (8-10 day-old) of wild-type *Ler* and mutant lines (*hrr*, HRR over-expression) were transferred into individual pots containing 4:1 mixture of soil (Siro) and vermiculite (Asfaltex). For the soil acclimation, the pots were covered with a plastic wrap to maintain the moisture level and kept in the growth room in the same conditions. After three days, the plastic wrap was removed and plants were watered every day, until approximately seven weeks of growth. When the siliques appeared desiccated (~ eight weeks), the seeds were harvested using a metallic sieve with a small mesh diameter for better separation from other senescent plant tissues. Harvested seeds were stored in the dark, at room temperature, in well-sealed individual tubes to prevent fungal contamination and rehydration.

3. Isolation of genomic DNA from Arabidopsis

Single leaves were placed in microtubes and ground to a fine powder with a micropestle and liquid nitrogen (N_2). To each tube, 500 µl of CTAB extraction buffer [2% (w/v) CTAB, 0.1 M Tris-HCl, pH 8.0; 1.4 M NaCl; 0.02 M EDTA, pH 8.0; 0.1% (v/v) 2-mercaptoethanol] were immediately added. Following an incubation at 65ºC (*Thermomixer,* Eppendorf), for 20 min (with agitation at each 5 min), an equal volume of chloroform was added. Microtubes were gently inverted and centrifuged at 13,000 rpm, for 5 min (*Heraeus Pico21 Centrifugue*, Thermo Scientific). Genomic DNA was precipitated by addition of 1 vol of chilled isopropanol. After gently swirling, the microtubes were centrifuged at 13,000 rpm, for 10 min. Precipitated DNA was washed by addition of 300 µl of 70% (v/v) ethanol and recovered by centrifugation. Finally, the resultant pellet was air-dried and resuspended in 25 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) and RNase treated (100 $\mu g.ml^{-1}$, Fermentas; during 30 min at 37ºC). The DNA was diluted to a final concentration of 100 $ng.\mu l^{-1}$ and stored at -20ºC.

4. Isolation of total RNA from Arabidopsis vegetative samples

Frozen vegetative tissue samples (50-100 mg) were ground in N_2 and homogenized with 1 ml of Trizol® reagent (Invitrogen). After homogenisation, samples were incubated at room temperature for 5 min and 200 µl of chloroform were added. The samples were vigorously shaked for 15 sec and then incubated for 2-3 min at room temperature. The samples were then centrifuged at 12,000 *g* for 15 min, at 4ºC (Sigma 2K15). Following centrifugation, aqueous phase was recovered and 500 µl isopropanol were added. The samples were gently mixed for 10 min and centrifuged at 12,000 *g* for 10 min, at 4ºC (Sigma 2K15). The resulting pellet was washed with 75% (v/v) ethanol. An additional centrifugation was performed (7,500 *g,* for 5 min, at 4ºC, Sigma 2K15) and RNA was air-dried for 15-20 min. Total RNA pellet was resuspended in 20 µl RNase-free water (pre-treated with DEPC, Sigma) and incubated for 10 min at 55-60ºC. RNA was kept at 4ºC (for immediate downstream procedures) or stored at -80ºC.

5. Isolation of total RNA from Arabidopsis seeds and silique samples

The total RNA from Arabidopsis seeds and siliques was extracted as described in Oñate-Sánchez and Vicente-Carbajosa (2008). Siliques, stratified and germinated seeds samples (100-250 mg) were ground in liquid N_2 and homogenised with 550 µl of extraction buffer [0.2 M Tris pH 8.0; 0.4 M LiCl; 25 mM EDTA, 1% (w/v) SDS]. After a briefly mix, 550 µl of chloroform were immediately added. The sample was vortexed during 15 sec and kept in ice until all samples were ready. After centrifugation (13,000 rpm, 3 min), the supernatant was transferred to a new microtube and 500 µl of water-saturated acidic phenol was added. The samples were thoroughly mixed and 200 µl of chloroform were added. After three to five minutes of incubation, with continuous and gently mixing, the samples were centrifuged at 13,000 rpm, during 3 min (Sigma 2K15). The supernatant (~600 µl) was transferred to a new microtube, cooled 8M LiCl was added to a final concentration of 2M and solution was mixed gently. To precipitate nucleic acids, the samples were incubated overnight, at 4ºC. The samples were then centrifuged at 16,000 rpm, during 30 min, at 4ºC (Sigma 2K15). The resulting pellet was dissolved in 22 µl of DEPC-water, added 3 µl buffer and 5 µl (5 U) of DNase I (Sigma). The incubation was taken into one hour, at 37ºC. The reaction was stopped by adding 5 µl of Stop solution (50 mM EDTA, Sigma), further incubated at 70ºC during 10 min and immediately chilled in ice. After five minutes of incubation in ice, 470 µl of DEPC-water, 7 µl of 3M NaAc pH5.2 and 750 µl of absolute ethanol was added. The samples were well mixed and centrifuged at 16,000 rpm, during 10 min at 4ºC to precipitate carbohydrates. The supernatant was transferred to a new microtube and 43 µl of 3M NaAc pH5.2 and 750 µl of absolute ethanol were added. The samples were well mixed and incubated at -20ºC for three to four hours and then were centrifuged at 16,000 rpm, during 20 min at 4ºC (Sigma 2K15). The resulting pellets were washed with 500 µl of 70% (v/v) ethanol and let to air-dry for 15-20 min. The RNA samples were resuspended in 20 µl of DEPC-water and kept at 4ºC (for immediate downstream procedures) or stored at -80ºC.

6. DNA and RNA quantification and quality

Nucleic acids quantification and purity were estimated by spectrophotometry using the *Nanodrop ND-100*. DNA or RNA concentration was determinated considering that an $A_{260\ nm}$ of 1 is equivalent to 50 µg DNA.ml^{-1} or 40 µg RNA.ml^{-1}. Purity was evaluated by $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios and quality was determined after fractioning DNA/RNA samples in agarose gel (section 7).

7. Agarose gel electrophoresis

DNA or RNA fragments were separated on an agarose gel [1-2% (w/v), prepared in 0.5x TAE buffer (40 mM Tris-HCl, pH 8.0; 20 mM acetic acid; 1 mM EDTA. pH 8.0)] stained either with ethidium bromide or Sybr Green (Invitrogen). Samples were pre-mixed with 6x Mass Ruler DNA Loading Dye (Fermentas) [10 mM Tris-HCl, pH 7.6; 0.03% (w/v) bromophenol blue; 60% (v/v) glycerol; 60 mM EDTA] and loaded onto the agarose gel. The ready-to-use Mass Ruler™ DNA Ladder Mix (Fermentas) was directly loaded. Electrophoresis was performed at 50-100 V, using a horizontal electrophoresis system filled with 0.5x TAE buffer. Fragments were visualised on an UV transilluminator (254 nm) and gels were revealed using the GenoSmart Imaging System (VWR) or ChemiDoc™ XRS (BioRad).

8. Polymerase Chain Reaction (PCR)

Amplification of DNA fragments was performed by PCR, either as described by Mullis and Fallona (1987) or following standard conditions described in user guides provided by manufacturer (*Platinum® Pfx DNA polymerase*, Invitrogen). PCR reaction conditions were prepared as referred in table B. PCR conditions are presented in annex IV.

Table B. Preparation of PCR reactions for standard/colony PCR and for cloning methodologies

	Standard/colony PCR	**Cloning PCR**
Template	500 ng^{-1} µg/ colony*	50 pg^{-1} µg
5x buffer *GoTaq* DNA polymerase (Promega)	10 µl	-
10x buffer *Pfx* DNA polymerase (Invitrogen)	-	5 µl
25 mM $MgCl_2$	4 µl	
50 mM $MgSO_4$	-	2 µl
10 mM dNTPmix	1 µl	1 µl
10 µM primers	2 µl (each)	2 µl (each)
GoTaq DNA polymerase (Promega) (1 U)	0.5 µl	-
Pfu DNA polymerase (1.25 U)	-	1 µl
Double distilled H_2O (ddH_2O)	up to 50 µl	up to 50 µl

*For performing PCR from *E. coli* transformed colonies: a single colony was scraped with a sterilised pipette tip and swirled into the PCR mixture. For *Agrobacterium* colonies: a single colony was first incubated in 20 mM NaOH, at 37ºC for 15 min; 5 µl of lysate was used as template in the PCR reaction.

9. First strand cDNA synthesis

After analysis of RNA concentration and quality, RNA was used as template for the first-strand cDNA synthesis using SuperScript First-Strand Synthesis System (Invitrogen). Total RNA (1 µg) was gently mixed with 0.5 µg of Oligo(dT)12-18 primer, 1 mM dNTP mix and DEPC-water up to a final volume of5 µl. After incubation at 65ºC for 5 min, and then placed on ice for at least 1 min, the cDNA synthesis reaction mixture was added (1x RT buffer, 5mM $MgCl_2$, 10 mM DTT, 20U RNase Out Recombinant RNase Inhibitor and 25U SuperScript II RT: Invitrogen). The reaction was incubated at 42ºC for 1.5 h, after which the reverse transcriptase was then deactivated at 85ºC, for 5 min and then kept at 4ºC. The reaction mixture was then treated with 1U of *E. coli* RNase H (Invitrogen) for 20 min, at 37ºC. The reactions were stored at -20ºC or kept at 4ºC to proceed immediately to RT-PCR amplification of cDNA.

10. Gene expression analysis by RT-PCR

RT-PCR was done as described in standard protocol for PCR reaction (section 8). Gene-specific primers pairs used for RT-PCR amplification was performed in same conditions using the constitutive *Actin2* gene (At3g18780, *ACT2*) and corresponding specific primers (Annex III). These primers have a position that span an intron region, important to detect genomic DNA contaminants. The number of cycles that fit into linear amplification zone of each analysed gene were previously optimised (Annex IV). The PCR products were analysed by agarose gel electrophoresis (section 7).

11. PCR fragments purification

The *Wizard SV Gel and PCR Clean-up System* (Promega) was used to purify PCR products directly from the PCR reactions and also from agarose gel, in accordance with the manufacturer instructions.

12. Gateway recombination reactions

Gateway BP and LR recombination reactions were prepared as presented in table C, for a final volume of 10 µl (according with the supplier instructions, Invitrogen). Both reactions were incubated at 25ºC, for 16-18 h. Recombination reactions were stopped with 1µl Proteinase K, at 37ºC, for 15 min.

Table C. Components used for the preparation of BP and LR recombination reactions

BP reaction	
attB PCR product	100 fmol
pDONR™201	100 fmol [1](~200 ng)
TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA)	Up to 8 µl
BP clonase™ II enzyme mix	2 µl
LR reaction	
Entry vector (pENTR)	200 ng
Destination vector (pDEST)	200 ng
TE buffer	Up to 8 µl
LR clonase™ II enzyme mix	2 µl

13. Enzyme restriction

Enzymatic reactions were performed in cloning strategy procedures, in attemping to obtain molecular constructs for *HRR* promoter activity analysis and for native expression of HRR.1 and HRR.2 fusion proteins. All components used in enzymatic reactions are depicted in table D. All reactions were incubated at 37ºC and stopped by adding application buffer (containing EDTA).

Table D. Components used for preparing enzyme reactions.

Cloning of HRR promoter for *pHRR::GFP6:HRR.1/ HRR.2* and *pHRR::HRR.1/ HRR.2:GFP6* constructs		
	***N*-termini fusion**	***C*- termini fusion**
pDNA	3 µg	3 µg
Buffer SuRE/Cut A (Roche)	2.5 µl	
Buffer SuRE/Cut M (Roche)		2.5 µl
BSA 10 $mg.ml^{-1}$	0.25 µl	
Shrimp Alkaline Phosphatase (SAP) (1U.μl^{-1}) (Fermentas)	1 µl	1 µl
*Hind*III (10U.μl^{-1}) (Roche)	1 µl	1 µl
*Kpn*I (10 U.μl^{-1})	1 µl	
*Spe*I (10 U.μl^{-1})		1 µl
Double distilled H_2O	Up to 25 µl	Up to 25 µl

[1] For the conversion of DNA femtomoles (fmol) to nanograms (ng)::$\boldsymbol{ng} = (\boldsymbol{fmol})(\boldsymbol{N})(\boldsymbol{660fg/fmol})(\boldsymbol{1\ ng/10^6 fg})$, where N is the size of DNA in bp, for BP reaction. [**N- HRR.1**(~560 bp); **HRR.2** (~633 bp, N-termini fusion or ~308 bp, C-termini fusion)]

Cloning *HRR* promoter in pCAMBIA1303 for *pHRR:: gusA* construct	
pDNA	4 µg
SuRE/Cut Buffer M (Roche)	2.5 µl
*Hind*III (10 U.µl^{-1}) (Roche)	1 µl
*Bgl*II (10 U.µl^{-1}) (Roche)	1 µl
SAP (1 U.µl^{-1}) (Fermentas)	1 µl
Double distilled H_2O	Up to 25 µl

14. DNA ligation

DNA ligation reactions between the plasmid and insert were performed using *T4 DNA ligase* (Roche), following the instructions provided by the supplier. The standard ligation reaction was set up considering a molar ratio of vector DNA to insert DNA of 1:3, following the next equation:

ng (insert)=ng (vector)*[(kb size (insert)/(kb size (vector)]*(molar ratio, insert:vector)

Linearised DNA vector and insert DNA were thoroughly mixed and diluted with 1 µl of 10x DNA ligation buffer, to a final volume of 10 µl. T4 DNA ligase (1 µl) was then added and gently mixed. The ligation reaction was incubated for 2 days, at 4ºC. The ligation reaction ixture was directly used for the transformation of *E. coli* competent cells (section 16).

15. Preparation of *Escherichia coli* competent cells

E. coli[1] cells were made chemically competent as performed by Inoue et al. (1990), with some modifications. A frozen aliquot (200 µl) of *E. coli* cells was spread into LB-agar plate, containing the selective antibiotic at recommended concentration, and grown overnight at 37ºC. A fresh colony was incubated in 8 ml SOB medium [2% (w/v) tryptone; 0.42% (w/v) yeast extract; 10 mM NaCl, 31 mM KCl, 10 mM $MgCl_2$, 5 mM $MgSO_4$] and grown overnight at 28ºC, with vigorous shaking (200 rpm). The overnight-grown culture was added to 200 ml SOB and grown at 28ºC, shaking vigorously at 200 rpm and until OD_{600} was about 0.6-0.7. The cells were shared in separate tubes and cooled on ice by 15-20 min. After incubation on ice, the cells were pelleted by centrifugation at 3,000 rpm, for 15 min at 4ºC (*Centrifugue 5804R*, Eppendorf). Each resulting pellet was resuspended in 17.5 ml cold TB buffer [10 mM PIPES; 15 mM $CaCl_2$; 250 mM KCl; after pH adjustment at 6.7, join 55 mM $MnCl_2$] and incubated on ice by 10 min. The cells were collected by centrifugation, in same

[1]Genotype of *E. coli* competent cells used: **XL1-Blue** (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*[F' *proAB lacI* ZΔM15 Tn*10*(*Tet*^{r})] (Bullock et al. 1987); ***ccd*B Survival T1R**, [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*x74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^r) *end*A1 *nup*G *ton*A::P_{trc}-*ccd*A]

conditions described previously. The cells were resuspended with 8 ml cold TB buffer and added DMSO, for a final concentration 7% (v/v). After incubation on ice by 20 min, 100 µl aliquots were made immediately frozen in N_2 and stored at -80ºC.

16. Transformation of E. coli competent cells

DNA (100 ng-1 µg) was gently added and mixed into an aliquot of ice-thawed competent cells and incubated for 25 min on ice. Cells were heat-shocked at 42ºC for 60 sec, with gentle agitation and immediately incubated on ice at least for 2 min. One millilitre of LB^+ medium [LB containing 0.4% (w/v) glucose and 20 mM $MgCl_2$] was added to the cell suspension. After incubation at 37ºC for one hour, with shaking at 200 rpm, cells were harvested (16,000 *g*, 1 min) (*Heraeus Pico21 Centrifugue*, Thermo Scientific) and resuspended in 100 µl of supernatant. Cells were spread on LB-agar medium supplemented with appropriate selection compound and incubated overnight at 37ºC.

17. Isolation of plasmid DNA

For preparing plasmid DNA for sequencing reactions, small-scale purifications were prepared using *GenElute™ HP Plasmid Miniprep Kit* (Sigma), according to supplier instructions. *E. coli* cells carrying the plasmid were firstly cultivated into 7 ml LB medium supplemented with the suitable antibiotic(s).

For cloning procedures, the phenol/chloroform/isoamyl alcohol (IAA) method (Serghini *et al.* 1989) was used, with some modifications. Firstly, a single *E. coli* colony was cultivated in 5 ml of LB medium containing the appropriate antibiotic(s) and grown was promoted during overnight at 37ºC, under continuous shaking (200 rpm). The *E. coli* culture was then spinned down by centrifugation at room temperature, at 16,000 *g* for 2 min (*Heraeus Pico21 Centrifugue*, Thermo Scientific). Pelleted cells were resuspended in 50 µl TEN buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA; 100 mM NaCl) and vortexing during 2 min. To lyse the cell suspension, 50 µl of phenol/chloroform/IAA (25/24/1, v/v/v) was added and briefly mixed using a vortex (2 sec). The lysate was centrifuged at 16,000 *g*, at room temperature for 10 min. After centrifugation, the aqueous layer was recovered and plasmid DNA was precipitated adding 17 µl of 7.5 M NH_4OAc and 100 µl isopropanol. After being thoroughly mixed, the suspension was centrifuged for 2 min, at 16,000 *g*. The pellet was rinsed with 1 ml of 70% (v/v) ethanol and let to dry for 20 min, in the flux chamber. The DNA pellet was resuspended with 20 µl of 10 mM Tris-HCl, pH 8.0. The final DNA solution was. digested with 100 $\mu g.ml^{-1}$ RNase A

(Fermentas), at 37ºC during one hour, and stored at -20ºC or kept at 4ºC for downstream procedures.

18. Preparation of *Agrobacterium tumefaciens* competent cells

A single colony of *A. tumefaciens* EHA105 (Hood et al. 1993) was inoculated into 5 ml LB medium supplemented with rifampicin (50 µg.ml^{-1}) and grown overnight at 28ºC with vigorous and constant shaking at 200 rpm. Next day, 100 µl of starter culture was diluted into 60 ml fresh LB medium and, under same incubation conditions referred above, the culture grown until a OD_{600}= 0.6-1. The cells were shared in tubes and left in ice by 10 min, before centrifugation step (3,000 *g*, at 4ºC for 6 min) (*Centrifugue 5804R*, Eppendorf). The settled cells were resuspended with one ml of ice-cooled 20 mM $CaCl_2$ (previously sterilized through 0.2 µm filter) and briefly centrifuged in same conditions as described above, for one min. Then the cells were resuspended in one ml of same solution and incubated on ice for 20 min. Aliquots of 100 µl *A. tumefaciens* were made, being either immediately frozen into N_2 and stored at -80ºC or used for transformation.

19. Transformation of *Agrobacterium tumefaciens* competent cells

DNA (500 ng- 5µg) was added to 100 µl frozen aliquot *A. tumefaciens* cells and let to thaw onto ice. The cell suspension was immediately frozen in N_2 for 30 sec and then incubated at 37ºC, for 5 min. After thawing, one ml LB medium was added and cells were incubated at 28ºC for three-four hours, with vigorous shaking at 200 rpm. After incubation, the cells were spread onto LB-agar medium supplemented with rifampicin plus the appropriate construct-selective antibiotic and incubated at 28ºC for two days.

20. Transformation of Arabidopsis plants by floral dip method

A. tumefaciens clones harbouring the appropriate construct were inoculated into five ml LB medium supplemented with rifampicin (50 µg ml^{-1}) plus the appropriate construct-selective antibiotic. Cell culture grown at 28ºC, with vigorous shaking at 200 rpm. Next day, one ml starter culture was diluted into 200 ml LB medium (pH 5.4), supplemented with construct-selective antibiotic and acetosyringone (19.6 µg.ml^{-1}) and incubated in same conditions referred as above, until OD_{600}= 0.6-0.8. Further, the cells were harvested by centrifugation at room temperature (5,000 rpm, for 15 min)

(*Centrifugue 5804R*, Eppendorf). The pelleted cells were ressuspended in MES buffer (10 mM MES pH 5.4, 10 mM $MgSO_4$) and briefly centrifuged in similar conditions mentioned previously. After removing supernatant, the pellets were ressuspended into 200 ml of 5% (w/v) sucrose, in which was further added 125 µl of 0.05% (w/v) Silwett L-77. The four-week-old plants (on early–middle bolting stage) were dipped into solution for one min, rolling gently to mix. Transformed plants were horizontally placed in plastic tray and covered with an opaque plastic by one day, in dark. After incubation, plants were transferred to the growth room, under standard conditions.

21. Generation and selection of transgenic plants

The *HRR* over-expression based constructs produced using the Gateway® cloning technology were used to transform wild-type *Ler* and *hrr* mutant plants. Transformation of Arabidopsis was achieved by floral dip method using *A. tumefaciens* strain EHA105 (section 20) (Clough and Bent 1998). Plant growth conditions of transformed plants (T0) and harvesting of their seeds were performed as described previously. Arabidopsis T1 seedlings germinate onto MS medium supplemented with 30 $\mu g.ml^{-1}$ hygromycin and 250 $\mu g.ml^{-1}$ ticarcilin until 10 days after sowing. The resistant seedlings were transferred to soil to obtain T2 seeds. Germinating these seeds onto same selective MS medium (40 $\mu g.ml^{-1}$ hygromycin and 250 $\mu g.ml^{-1}$ ticarcilin) allowed verifying if the transgene was segregating under a Mendelian purpose. Once proved, a single insertion of transgene would have occurred. With their seeds (T3) a new screening in same MS-selective medium was performed to determine the genotype of individual T3 plants (ratio 1:0), using them for further experiments.

ANNEX III: OLIGONUCLEOTIDE SEQUENCES

The oligonucleotides (primers, Table E) were synthesized by *Metabion* Services (Germany). Stock primers solutions were prepared to a final concentration of 100 µM in double-distilled water, according with suppliers instructions. A working solution of 10 µM was used for PCR amplification.

Table E. Oligonucleotides used in this work for each purpose. The recombinant Gateway sequences and enzyme restriction sites are underlined.

	Sequence (5' to 3')
Genotyping for *hrr* mutants	
HRR_LP	TTCTAGGGCTCAGATTGTTCG
HRR_RP	ATCCCGGTGTCGAAAGAAACT
prbZF_Rv	ACTTGCAAAGTCCCGCTAGT
***HRR* promoter amplification, for p*HRR* fusion constructs**	
PromotorHRR_fwd (*Hind*III)	ACGAAGCTTGCAGATGAAGCAAGAAAAAGGGAA
PromotorHRR_rv (*Bgl*II)	CCTCTAGATGGTACAGAGAAACCTTTCATTTTCTT
PromotorHRR_rv2 (*Kpn*I)	GAGGTACCTCTCTTTGGAAAGTAAAAGAAAGGT
PromotorHRR_rv3 (*Spe*I)	GAACTAGTTCTCTTTGGAAAGTAAAAGAAAGGT
HRR cDNA amplification (Gateway cloning)	
HRRcDNA_fw	ATGTCTCACCACCACCAAAACT
HRRcDNA_rv	TTAGCGAAGATCCCGGTGTC
HRR GC1	AAAAAGCAGGCTTAGACATGTCTCACCACCACCAAAAC
HRR GD1(HRR.1 and HRR.2, N-terminal fusion)	AGAAAGCTGGGTGTTAGCGAAGATCCCGGTGTCGAA
HRR GE1 (HRR.1, C-terminal fusion)	AGAAAGCTGGGTAGCGAAGATCCCGGTGTCGAAAG
HRR GC2	AAAAAGCAGGCTTAGACATGTCTCACCACCACCA
HRR GE2 (HRR.2, C-terminal fusion)	AGAAAGCTGGGTAAACGTGACCTGAAGAAAGATATA
Gateway BP entry primers (*att*B adapters)	
attB1(Fw)	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2 (rv)	GGGGACCACTTTGTACAAGAAAGCTGGGT
Verification of *pENTR* vectors	
pdon201 Seq Fw	TCGCGTTAACGCTAGCATGGATCTC
pdon201 Seq Rv	GTAACATCAGAGATTTTGAGACAC

Verification of *pHRR::gusA* vectors (via *pCAMBIA1303*)	
L35#2	TTGGCCGATTCATTAATG
R 35S	AGTTTTTTGATTTCACGG
Verification of Gateway LR cloning reaction; genotyping of HRR over-expression transgenic plant lines	
pMDC 35S	TTCATTTCATTTGGAGAGGACC
pMDC gfp left	TTGGGACAACTCCAGTGAAAAG
pMDC gfp right	GGATTACACATGGCATGGATG
pMDC32 R2 Flank	CGGCCGCTCTAGAACTAGTTAA
T35S	TGATTTTTGCGGACTCTAGC
P35S	TTCATTTGGAGAGGACTCCGGTA
eFP.R	GATCACATGGTCCTGCTGGA
eFP.L	CAGCTCGACCAGGATGGGCAC
RT-PCR (HS-dependent expression analysis)	
ACT2_F2	AAGATCTGGCATCACACTTTCT
ACT2_R2	ATGGCATGAGGAAGAGAGA
HRR_RT_fw	ATGTGAACGTCGTTTGTGAT
HRR_RT_rv	CGGTGTCGAAAGAAACTCTG
HsfA2_fw	CCACGTTACTTCAAGCATAGCA
HsfA2_rv	AAGGTTCCGAACCAAGAAAAC
Hsp18.1_fw	TGTCTCTCATTCCAAGCATTTTTGG
Hsp18.2_rv	TAGCCCCGGAGATATCGATGGAC
Hsp25.3_fw	TCTAACATTTGTCGCATCGC
Hsp25.3_rv	CGGCTCTATGTTCACCTCTT
Hsa32_fw	TGGCGGCTTACTACAGATGGAAG
Hsa32_rv	TAAAACAAGAAGTAGGAGGAACTGAG
HOT1_fw	TCATCTTCTTCACCGCCTGA
HOT1_rv	TCTGTCTCTCGCCTCATTGG

RT-PCR (Seed development and germination, including HS treatment)	
Abi3_RTfw	ACAACAACAACAACAATAATCAGC
Abi3_RTrv	CTTACTTTAACCCCTCGTATCAA
Abi4_RTfw	ATAACCCGGATCCAGACCCATAG
Abi4_RTrv	TACCGTGGCGTTCGACAAAGAAG
Abi5_RTfw	GACAACTCGGGTTCCTCATCAAT
Abi5_RTrv	CGTTAAGCCCGGTGTCTTCAGAT
LEC1_RTfw	TTCCGTTAATGGAAGACGAAGAG
LEC1_RTrv	TGGAGCTCCCTTCTCTCACTATC
LEC2_RTfw	TCACCACCACTCAAAGTCGTTAA
LEC2_RTrv	GATAACTTCTTACCCTTTCCCTC
FUS3_RTfw	TTCTCAACGGAGCCCAAACCATC
FUS3_RTrv	ATCATCATGGGTTATCGGCGTCT
EM6_RTfw	TTAGGTCTTGGTCCTGAATTTGG
EM6_RTrv	ATGGCGTCTCAACAAGAGAAGAA
EM1_RTfw	ATGGCGTCAAAGCAACTGAGCAG
EM1_RTrv	TCTCCACCAGATTTTTCCATCGT
HSFA9_RTfw	GGCAATTCCAAACGTCGTCGATA
HSFA9_RTrv	CTCGTATTCCCGCTTTGATTGTT
NCED9.fw	CGATCCATTGAATCGACGAAG
NCED9.rv	TTCCGGTGACTGGAACAGTAC
ABA1.fw	TATTTGGCTGCATCGCATGGA
ABA1.rv	ACGCTTTAGCTGCTTTGGAAG
CYP707A1.fw	GATATCTCCGCCTTGTTTCTC
CYP707A1.rv	AAGCGACTCTTAATGTTTCTT
GA3ox1.fw	CCGATTGGTATAGAGGCGATT
GA3ox1.rv	CTTCTCCGCTGCTCCTTCTCC
SPY.fw	TGCCCTTACACACTGTGGTAT
SPY.rv	TGTAAAGTACTCCCAAATTGT

ANNEX IV: PCR CONDITIONS

Amplifications by PCR were performed using *MJ Mini Gradient Thermal Cycler* (Bio-Rad) or *Mastercycler Gradient* (Eppendorf) under the conditions described below (Table F).

Table F. PCR conditions used for genotyping, screening of cloned colony, Gateway cloning steps, cloning of HRR promoter and semi-quantitative RT-PCR.

	Temperature (time)	Number of cycles
Genotyping and colony PCR		
Initial Denaturation	94ºC (5 min)	
Denaturation	94ºC (45 sec)	31
Annealing	56ºC[a] and 60ºC[b] (45 sec)	
Extension	74ºC (45s -1min 30s)	
Final extension	74ºC (5 min)	
Gateway- based cloning, 1st PCR		
Initial Denaturation	95ºC (2 min)	
Denaturation	95ºC (40 sec)	35
Annealing	55ºC[c] and 60ºC[d] (40 sec)	
Extension	74ºC (1min 30sec)	
Final extension	74ºC (5 min)	
Gateway- based cloning, 2nd PCR		
Initial Denaturation	95ºC (2 min)	
Denaturation	95ºC (40 sec)	5
Annealing	45ºC (40 sec)	
Extension	74ºC (1min 30sec)	
Denaturation	95ºC (40 sec)	25
Annealing	55ºC[c] and 60ºC[d] (40 sec)	
Extension	74ºC (1min 30sec)	
Final extension	74ºC (5 min)	
Cloning of *HRR* promoter (p*HRR*)		
Initial Denaturation	95ºC (2 min)	
Denaturation	95ºC (40 sec)	35
Annealing	53ºC (40 sec)	
Extension	74ºC (1min 30sec)	
Final extension	74ºC (5 min)	
RT-PCR		
Initial Denaturation	94ºC (5 min)	
Denaturation	94ºC (40 sec)	*n* cycles[e]
Annealing	55 - 58.5ºC (40 sec)	
Extension	74ºC (1min 30sec)	
Final extension	74ºC (5 min)	

a) *hrr* mutants and wild-type *Ler;* Verification of transformants, ectopic expression of HRR in fusion proteins

b) HRR over-expression lines; Verification of transformants, native expression of HRR in fusion proteins

c) *HRR.1* sequences (N- and C-termini fusions); *HRR.2* sequence for C-termini fusion

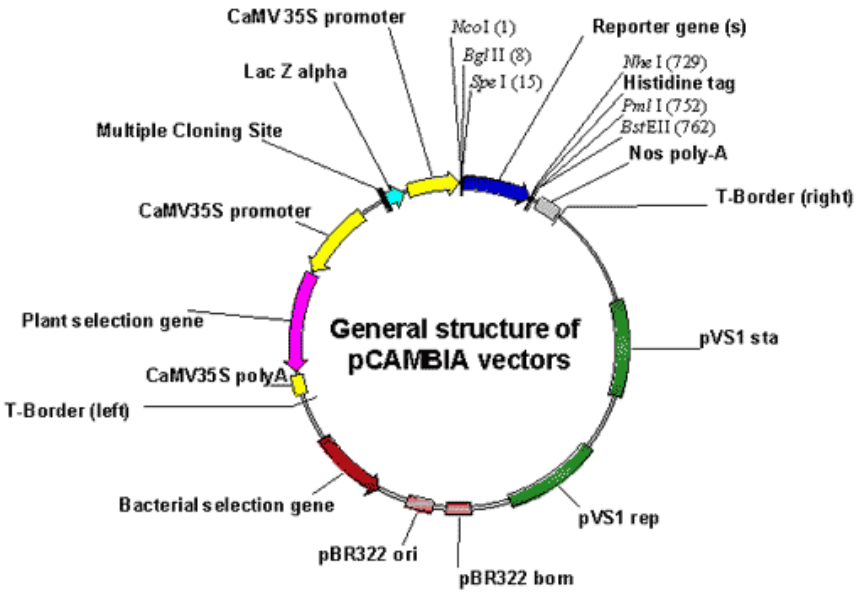
d) *HRR.2* sequence (N-termini fusion)

e) **Expression analysis during maturation and germination processes**: *HRR* (33), *ABI3* (30), *ABI5* (31), *FUS3* (31), *LEC1* (33), *LEC2* (40), *EM1* (29), *EM6* (25), *ABI4* (35), *HsfA9* (30), *ABA1* (30), *NCED9* (33), *CYP707A1* (31), *GA3ox1* (31), *SPY* (32); **Expression analysis in HS-stressed wild-type *Ler*, *hrr* and HRR over-expression mutant seed lines**: *HsfA2* (31), *Hsp101*(27), *Hsa32* (33), *Hsp25.3* (36), *Hsp18.1*(32), *ABI3* (31), *ABI5* (31), *FUS3* (33), *LEC1* (33), *EM1* (25), *EM6* (25), *ABI4* (35), *HsfA9* (30), *ABA1* (30), *NCED9* (31), *CYP707A1* (31), *GA3ox1* (31), *SPY* (32).

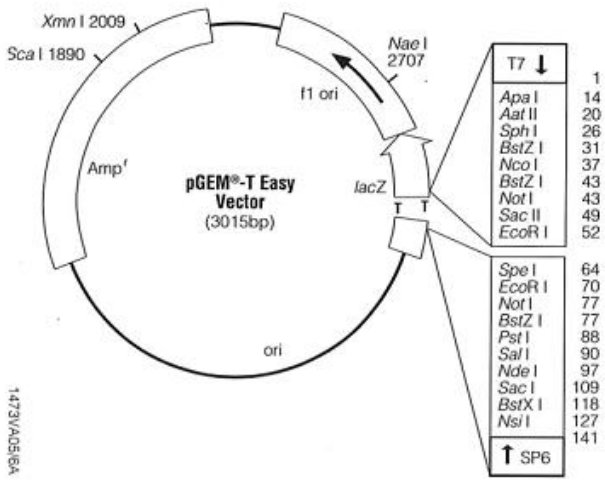
ANNEX V: BASE VECTOR MAPS

Maps of the vectors used during the cloning procedures.

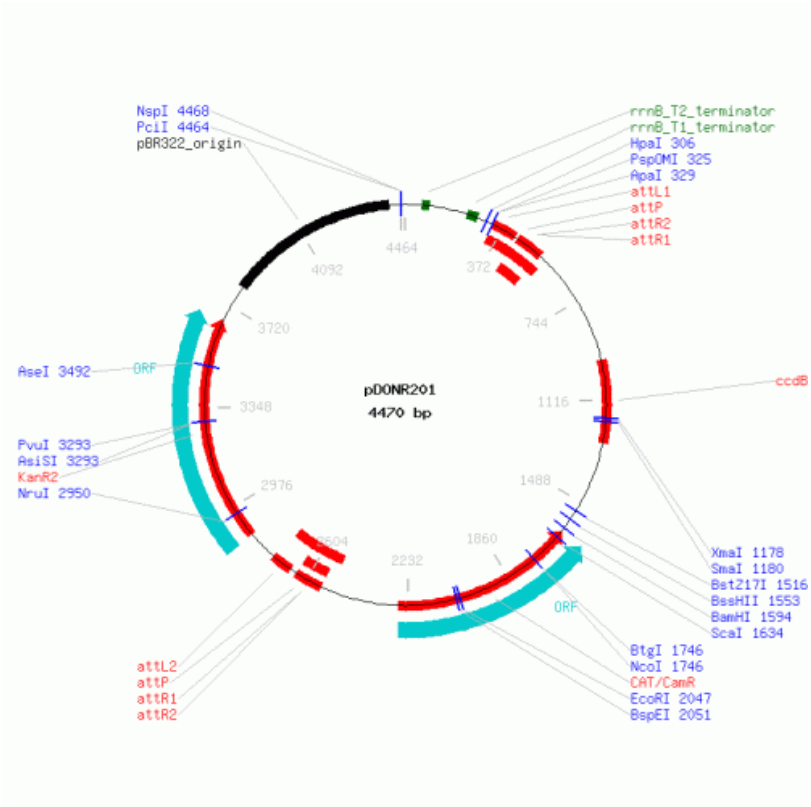
pCAMBIA1303



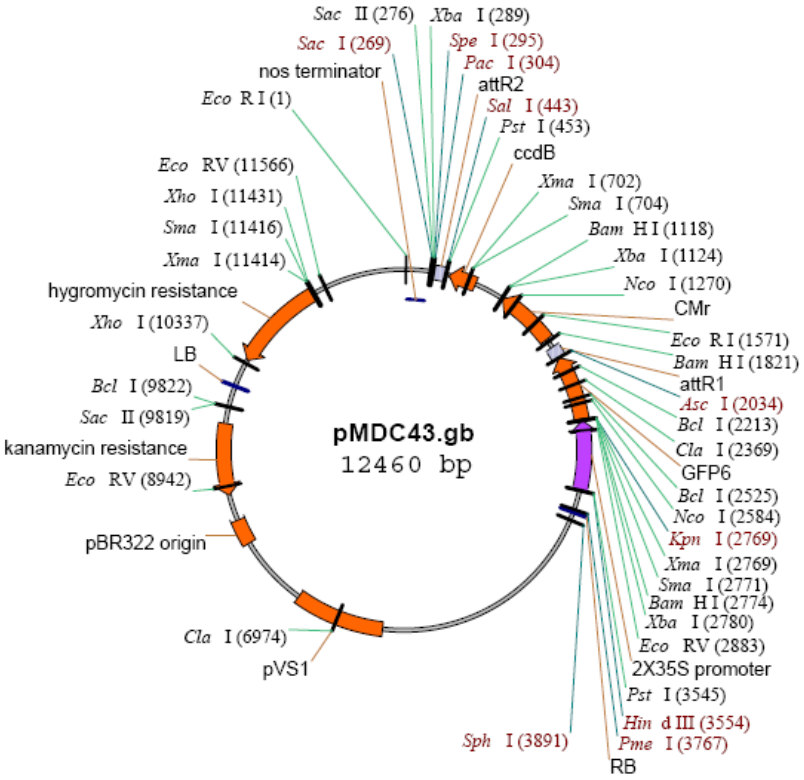
pGEM® T-easy vector system (Promega)



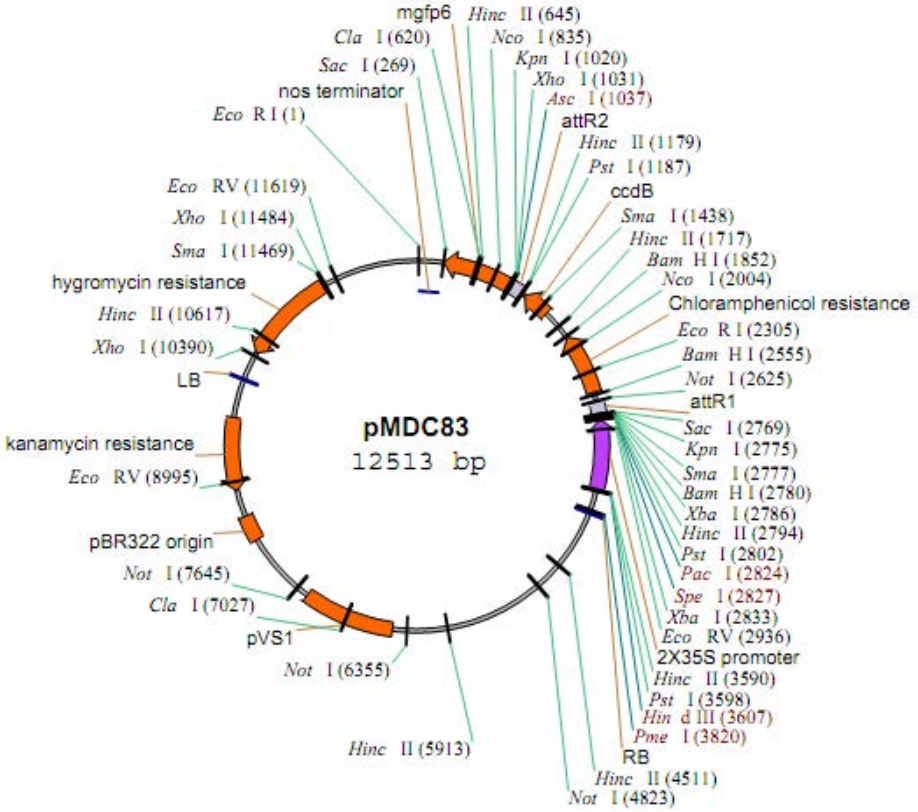
pDONR™ 201



pMDC43



pMDC83


pMDC83
12513 bp
mgfp6
Hinc II (645)
Cla I (620)
Nco I (835)
Sac I (269)
Kpn I (1020)
nos terminator
Xho I (1031)
Asc I (1037)
Eco R I (1)
attR2
Hinc II (1179)
Pst I (1187)
Eco RV (11619)
ccdB
Xho I (11484)
Sma I (1438)
Sma I (11469)
Hinc II (1717)
Bam H I (1852)
hygromycin resistance
Nco I (2004)
Hinc II (10617)
Chloramphenicol resistance
Xho I (10390)
Eco R I (2305)
LB
Bam H I (2555)
Not I (2625)
attR1
kanamycin resistance
Sac I (2769)
Kpn I (2775)
Eco RV (8995)
Sma I (2777)
Bam H I (2780)
Xba I (2786)
pBR322 origin
Hinc II (2794)
Pst I (2802)
Not I (7645)
Pac I (2824)
Cla I (7027)
Spe I (2827)
Xba I (2833)
pVS1
Eco RV (2936)
2X35S promoter
Not I (6355)
Hinc II (3590)
Pst I (3598)
Hin d III (3607)
Hinc II (5913)
Pme I (3820)
RB
Hinc II (4511)
Not I (4823)

ANNEX VI: CLONING STRATEGY

The production of *HRR* transgenes for subcellular localisation studies was performed based on Gateway cloning technology (Invitrogen). However, some modifications were introduced in the system of destination vectors. It was performed a substitution of dual 35S CaMV promoter (in pMDC43) by *HRR* promoter sequence, in attemping to obtain the native expression of HRR proteins. The main steps of the cloning strategy used in this work are depicted in Figure A1.

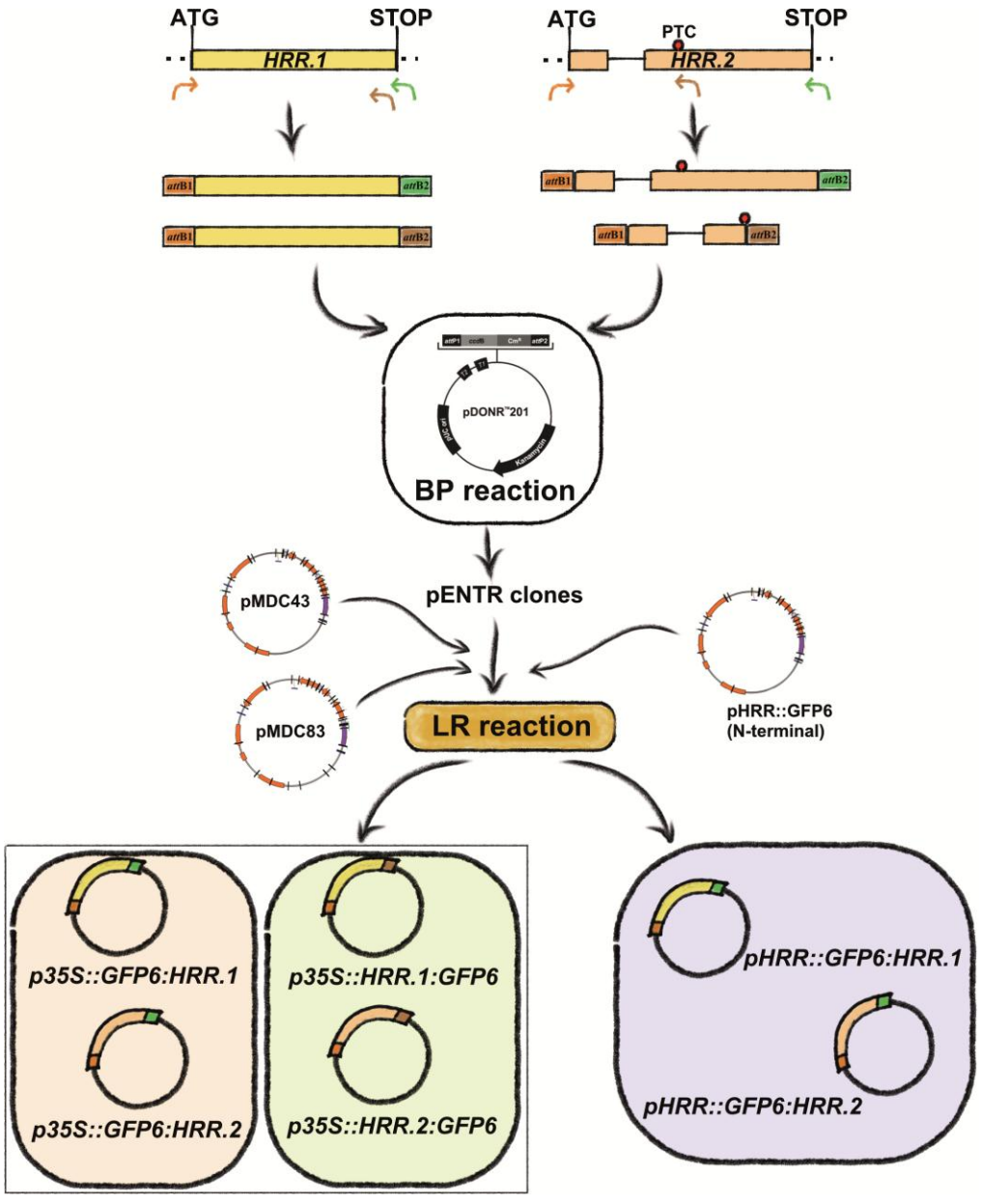


Figure A1. Overview of the cloning strategy based on Gateway® technology used to obtain *HRR.1* and *HRR.2* fusion transgenes. The fragments of interest were amplified by two-rounds of PCR amplification, in attempting to create *att*B sites in both ends [*att*B1-orange, *att*B2- green (with STOP codon, for N-termini fusion) and brown (without STOP codon, for C-termini fusion)]. The fragment of interest was inserted into the donor vector (pDONR™201) by the BP recombination reaction. A subsequent LR recombination reaction promoted the insertion of the DNA sequence into the appropriate destination vector (pMDC vectors). The generated expression clones were used in plant transformation (p*35S::HRR.1:GFP6*) and in subcellular localisation studies.