Universidade do Minho
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Molecular Mechanisms Associated to Thermotolerance in Plants

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Molecular Mechanisms Associated to Thermotolerance in Plants

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"Research is to see what everybody else has seen,
and to think what nobody else has thought."
Albert Szent-Györgi

Aos meus pais

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MOLECULAR MECHANISMS ASSOCIATED TO THERMOTOLERANCE IN PLANTS

ABSTRACT

One of the most typical abiotic stresses encountered by plants is extreme temperatures. High temperature leads to a series of morphological, physiological and molecular alterations that adversely affect plant growth and productivity. Acquisition of thermotolerance is largely controlled through molecular mechanisms based on the activation and regulation of specific stress-related genes. The elucidation of these gene/protein functions will give insights on the various mechanisms of plant response to heat stress, providing useful information to improve plant thermotolerance. The present work aims to contribute for the understanding of the molecular mechanisms that are responsible for plant adaptation to heat stress. Two species, *Populus euphratica* Oliv. and *Arabidopsis thaliana* L., were used as models due to the latest development of genomic and molecular biology resources and tools for both plants.

P. euphratica is naturally found under severe conditions such as extreme temperatures (-45°C to +54°C), high soil salinity and drought. The physiological response of *P. euphratica* cultured cells was evaluated at different temperatures. Contrasting with its innate enhanced tolerance to extreme temperatures, the *in vitro* system did not present an outstanding tolerance capacity. *P. euphratica* suspended cells heat-shocked for 20 min were able to tolerate temperatures up to 45°C. Heat-associated events as PCD and ROS production were suggested not to be implicated in the occurring cell death.

Supported by the use of publicly available *A. thaliana* expression data and other web-based tools and resources, a reverse genetics strategy was followed for the identification of novel determinants for heat stress tolerance (*HZF* and *HRR*). *In silico* analysis revealed that both genes putatively encode effector proteins involved in different stages of the heat stress response. Moreover, *HZF* and *HRR* were found to be co-regulated with genes already implicated in the regulation of heat responses. Functional characterization of *HZF* was primarily supported by the use of several web-based tools and resources specifically created for *Arabidopsis* functional analysis. HZF was found to be a zinc finger family protein containing a conserved C3H2C3-type RING domain and its possible role as E3 ubiquitin ligase was suggested.

To pursue with reverse genetics approaches for identifying heat stress-associated mutations, a phenotypic analysis based on germination and seedling survival assays was proposed. Temperatures and periods of treatment were diversely combined to test basal thermotolerance in either seeds or 7-day-old seedlings, or acquired thermotolerance only in 7-day-old seedlings. The effectiveness of the proposed protocols was illustrated by detection of heat-associated phenotypes in two mutants (*hot1-3* and *atrbohD*) previously identified to be thermotolerance defective. Regarding germination assays, special attention should be given to the time-course evaluation of the number of germinated seeds for an accurate phenotypic detection. A delayed germination was observed in *hzf* mutant seeds in the following days after heat treatment when compared to wild-type seeds, suggesting a role for HZF in the transition from dormant to germinating state. HZF was then suggested to mediate the ubiquitination of a regulator protein implicated in promoting seed dormancy or repressing germination upon heat stress. This function seems to be mainly assured by a redundant gene product (L-HZF) under standard conditions, since similar germination timing was observed for *hzf* and wild-type seeds. Maximum *HZF* transcript accumulation in heat-treated (38°C for 1 h) wild-type seedlings was achieved 15 min after heat treatment, suggesting also the HZF involvement in the initial phase of heat stress response. Expression vectors suitable for overexpression studies and *in situ* analysis were constructed and used to transform wild-type *Arabidopsis* plants. The transgenic T3 plants will be soon available for further experiments that will contribute to elucidate the specific role of *HZF* in thermotolerance. The complete functional characterization of *HZF*, currently in progress, will provide novel information that would contribute to the dissection of its particular role in plant thermotolerance.

KEYWORDS *Arabidopsis thaliana* · Functional genomics · Heat stress responsive genes · *Populus euphratica* · Reverse genetics · Thermotolerance

MECANISMOS MOLECULARES ASSOCIADOS À TERMOTOLERÂNCIA EM PLANTAS

RESUMO

A temperatura extrema constitui um dos principais factores de stresse abiótico a que as plantas estão frequentemente sujeitas. A exposição a temperaturas elevadas resulta em alterações profundas do metabolismo, executadas a nível morfológico, fisiológico e molecular, que prejudicam o desenvolvimento e produtividade vegetal. A aquisição de termotolerância é essencialmente controlada por mecanismos moleculares, os quais incluem a activação e regulação de genes específicos associados ao stresse. A elucidação da função desses genes/proteínas irá contribuir para a compreensão dos mecanismos de resposta das plantas que permitem a aquisição de tolerância a temperaturas elevadas. O conhecimento desses mecanismos poderá ainda ser utilizado na implementação de estratégias para o aumento da termotolerância em plantas. O presente trabalho pretende contribuir para o conhecimento dos mecanismos moleculares responsáveis pela adaptação ao stresse térmico em plantas. Duas espécies, *Populus euphratica* Oliv. e *Arabidopsis thaliana* L., foram utilizadas como modelos, devido ao recente desenvolvimento de recursos e ferramentas genómicas e de biologia molecular.

P. euphratica encontra-se distribuída em ambientes adversos, designadamente apresentando temperaturas extremas (-45°C a +54°C), elevada salinidade e secura. Neste trabalho, procedeu-se à caracterização fisiológica de células em suspensão de *P. euphratica* a diferentes temperaturas. Contrariamente à elevada termotolerância inata, o sistema *in vitro* não apresentou termotolerância significativa. As células em suspensão de *P. euphratica*, sujeitas a stresse térmico durante 20 min, atingiram um máximo de tolerância para temperaturas inferiores a 45°C. A avaliação de processos associados à resposta a temperaturas elevadas, como a morte celular programada ou a produção de espécies reactivas de oxigénio, sugeriu que estes não se encontram implicados na morte celular observada.

Fazendo uso de informação sobre expressão genética e outras ferramentas e recursos disponibilizados publicamente para a espécie *A. thaliana*, foi efectuada a identificação de novos determinantes para a tolerância ao stresse térmico (*HZF* e *HRR*). A análise *in silico* revelou que ambos os genes possivelmente codificam proteínas efectoras envolvidas em diferentes estádios da resposta ao stresse térmico. A análise de genes que apresentam co-

expressão com *HZF* e *HRR* permitiu identificar outros genes implicados na regulação de respostas a temperaturas elevadas. A caracterização funcional do gene *HZF* foi inicialmente suportada pelo uso de ferramentas e recursos disponíveis *on-line* especialmente concebidos para análise funcional em *Arabidopsis*. HZF foi identificado como sendo uma proteína da família *zinc finger*, que possui um domínio conservado do tipo RING C3H2C3, tendo sido sugerida a sua possível função como E3 ubiquitina ligase.

De forma a prosseguir a metodologia de genética inversa, para a identificação de mutações associadas ao stresse térmico, foi proposta uma análise fenotípica baseada em ensaios de germinação e viabilidade de plântulas de *A. thaliana*. Os protocolos utilizados abrangeram uma combinação de temperaturas e tempos de tratamento térmico, para avaliar a termotolerância basal em sementes e plântulas com 7 dias, ou a termotolerância adquirida em plântulas com 7 dias. A eficácia dos protocolos foi demonstrada através da identificação de fenótipos associados ao calor em dois mutantes (*hot1-3* e *atrbohD*), cuja termotolerância reduzida tinha sido previamente descrita. No que se refere aos ensaios de germinação, foi sugerida a avaliação do número de sementes germinadas ao longo do tempo para uma detecção fenotípica mais precisa. Quando comparadas com as sementes da estirpe selvagem, as sementes do mutante *hzf* exibiram atraso na germinação nos dias seguintes ao tratamento térmico, sugerindo o envolvimento de HZF na transição na fase de dormência para o estado germinativo. A proteína HZF foi então sugerida como mediadora da ubiquitinação de uma proteína reguladora implicada na manutenção da dormência ou repressão da germinação em condições de stresse térmico. Esta função parece ser principalmente assegurada por uma outra proteína com função redundante (L-HZF) em condições normais, dado que o tempo de germinação observado para as sementes *hzf* e selvagem foi semelhante. A máxima acumulação de transcriptos de *HZF* em plântulas selvagem sujeitas a stress térmico (38°C durante 1 h) foi observada 15 min após tratamento, o que sugeriu também o envolvimento de HZF na fase inicial da resposta ao stresse térmico. Vectores de expressão apropriados para estudos de sobre-expressão e análise *in situ* foram concebidos e utilizados na transformação de plântulas selvagem de *A. thaliana*. As plantas transgénicas T3 estarão brevemente disponíveis para ensaios suplementares que irão contribuir para esclarecer a função específica de *HZF* na termotolerância. A caracterização funcional completa do gene *HZF*, actualmente em curso, irá fornecer informação adicional que poderá contribuir para a elucidação do seu papel particular na termotolerância de plantas.

PALAVRAS-CHAVE *Arabidopsis thaliana* · Genes de resposta ao stress térmico · Genética inversa · Genómica funcional · *Populus euphratica* · Termotolerância

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Chapter 1

General introduction – Heat response and tolerance in plants

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TEMPERATURE STRESS – A MAJOR ABIOTIC STRESS IN PLANTS

Plants are continuously exposed to an extensive array of environmental insults which result in the loss of agricultural productivity (Boyer 1982; Bray *et al.* 2000). Abiotic stress is caused by the occurrence of individual or combined detrimental conditions, such as drought, salinity, extreme temperatures or chemical toxicity (Figure 1.1) (reviewed by Wang *et al.* 2003). Lacking locomotion as a mean to escape dramatic changes in their environment, the range of behavioural responses by which plants are able to avoid stress is limited (Wahid *et al.* 2007). However, plants have evolved a variety of sophisticated responses that enable them to tolerate and survive in adverse conditions, including the long- and short-term morpho-anatomical adaptations, like the alteration of leaf orientation or membrane lipid composition (reviewed by Wang *et al.* 2003; Madhava Rao *et al.* 2006). As part of their adaptive mechanisms to cope with stress, plants also display several physiological and molecular adjustments. Therefore, plant adaptation to abiotic stresses comprises a series of morphological, physiological and molecular changes, which are all controlled by complex molecular networks (reviewed by Wang *et al.* 2003; Madhava Rao *et al.* 2006).

In plant stress studies, primary stresses (*e.g.*, drought, salinity, extreme temperatures or chemical toxicity) have been distinguished from secondary stresses that result from the impact of the former. Osmotic and oxidative stresses arise often as a consequence of a primary stress or a combination thereof (Figure 1.1). For example, oxidative stress frequently accompanies high temperature, salinity or drought stress. Thus, the way in which a plant senses and responds to different abiotic factors seems to be related and may lead to similar damage and responses at the cellular and molecular level. In contrast, it has been shown that a combination of two different environmental stresses could result in an unique response, dissimilar from the

KEYWORDS Abiotic stress · Functional genomics · Heat response · Temperature perception · Thermotolerance

ABBREVIATIONS ABA: **ab**scisic **a**cid · ACC: 1-**a**mino**c**yclopropane-1-**c**arboxylic acid · CDPK: **c**alcium **d**ependent **p**rotein **k**inases · HSE: **h**eat **s**hock **e**lement · HSF: **h**eat **s**hock transcription **f**actor · HSP: **h**eat **s**hock **p**rotein · LEA: **l**ate **e**mbryogenesis **a**bundant · MAPK: **m**itogen **a**ctivated **p**rotein **k**inases · PCD: **p**rogrammed **c**ell **d**eath · ROS: **r**eactive **o**xygen **s**pecies · SA: **s**alicylic **a**cid

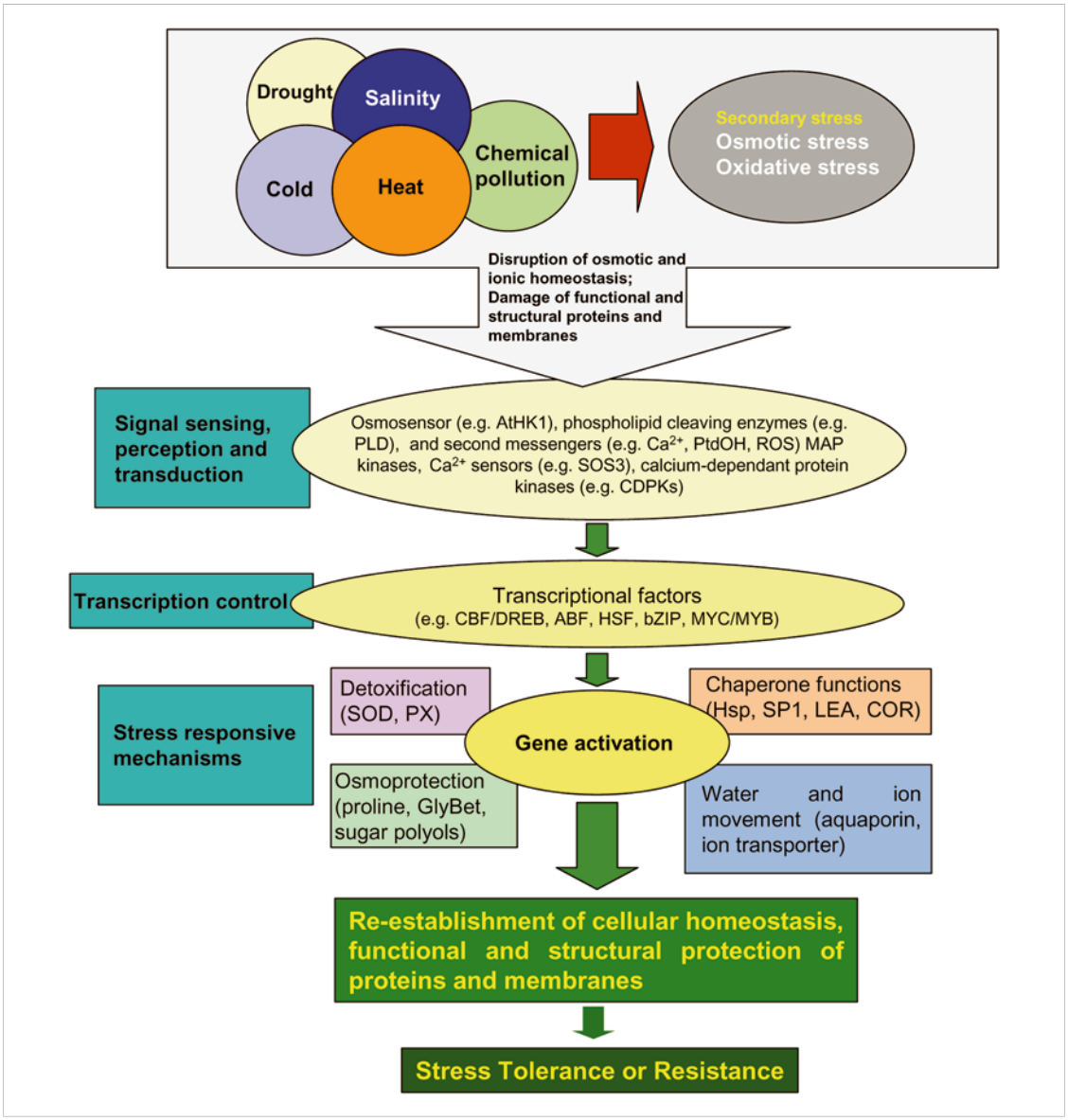

Figure 1.1

Molecular events associated with plant response to abiotic stress. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected and lead to osmotic and oxidative stress, which can be considered as secondary stresses. As a result, diverse environmental stresses often activate similar cell signaling pathways. The initial stress signals (e.g. osmotic and ionic effects, temperature or membrane fluidity changes) are perceived and trigger downstream signaling processes and transcription control. Stress-responsive mechanisms are then activated to re-establish homeostasis and protect and repair damaged proteins and membranes (Wang *et al.* 2003).

ABF: **AB**A-responsive element binding **f**actor; AtHK1: ***A**rabidopsis **t**haliana* **h**istidine **k**inase-**1**; bZIP: **b**asic leucine **zip**per transcription factor; CBF/DREB: **C**-repeat-**b**inding **f**actor/**d**ehydration-**re**sponsive **b**inding protein; CDPK: **c**alcium-**d**ependent **p**rotein **k**inase; COR: **co**ld-**r**esponsive protein; Hsp: **h**eat **s**hock **p**rotein; LEA: **l**ate **e**mbryogenesis **a**bundant; MAP: **m**itogen-**a**ctivated **p**rotein; PLD: **p**hospho**l**ipase **D**; PtdOH: **p**hospha**tid**ic acid; PX: **p**ero**x**idase; ROS: **r**eactive **o**xygen **s**pecies; SOD: **s**uper**o**xide **d**ismutase; SP1: **s**table **p**rotein **1**

response to each individual stress (Mittler 2006). The complexity of the mechanisms leading to tolerance makes the task of understanding plant responses to abiotic stresses extremely difficult.

The immediate focus of plant stress research has been to understand the molecular events and the regulatory pathways that occur in response to stress (Wang *et al.* 2003; Bhatnagar-Mathur *et al.* 2008). After the initial perception of stress, a signal transduction cascade is triggered, leading to the activation of protective mechanisms by alterations in the expression of stress-related genes and metabolites (Figure 1.1). The control of abiotic stress tolerance is based on the activation and regulation of specific genes involved in signaling, transcription regulation, protection of membranes and proteins (including those with chaperone function, ROS scavengers and osmoprotectants) and water and ion influx and transport (reviewed by Wang *et al.* 2003; Vinocur and Altman 2005). Although recent advances in understanding these responsive mechanisms have been achieved, the complex regulatory networks underlying abiotic stress tolerance are still far from being elucidated (reviewed by Sreenivasulu *et al.* 2007).

One of the most common primary stresses that plants have to face from their surroundings is temperature stress (Sung *et al.* 2003). The optimum temperature for growth is intrinsic to each plant species and establishes its geographical distribution, according to the temperature zone in which plant can survive (Iba 2002). Plants can experience three types of temperature stresses: a) temperatures below freezing, b) low temperatures above freezing, and c) high temperatures. Low and high temperatures share some similar responses, being the most important the alteration of membrane fluidity, production of compatible solutes and heat shock proteins (HSP) and a slowdown in metabolism and energy dissipation (Sung *et al.* 2003; Beck *et al.* 2007). These alterations ultimately result in radical formation and oxidative stress, with the consequent activation of the antioxidant system. However, at the level of temperature perception and signaling, it seems that the temperature signal is transduced by non-overlapping and independent pathways.

As global climate seems to be definitively tending to increase the average temperatures, the study of heat tolerance mechanisms is therefore of major importance (reviewed by Wahid *et al.* 2007). Understanding the physiological and

molecular mechanisms of plant adaptation to heat stress would provide the basis for the production of heat-tolerant species with improved productivity.

PLANT RESPONSES TO HEAT STRESS

Temperature elevation above the normal optimum (usually 10-15°C above ambient temperature) is perceived by plants as heat stress (Wahid *et al.* 2007). The rate, intensity and duration of temperature increase determines the severity of the imposed stress and the corresponding effects on physiological and metabolic processes (Sung *et al.* 2003). At extreme high temperatures, severe injuries and cell death may occur immediately. At moderately high temperatures, immediate effects include denaturation and aggregation of proteins and increased fluidity of cellular membranes. Only after long-term exposure, this moderate stress may eventually lead to cell death.

An extensive diversity of structures and functions is negatively affected by heat causing different morphological, physiological and molecular responses, which can lead to plant adaptation (reviewed by Wahid *et al.* 2007). Some of the most pertinent plant responses to heat stress are considered bellow (Figure 1.2).

Morphological responses

Plant growth and productivity are highly adversely affected by high temperatures at all developmental stages (Madhava Rao *et al.* 2006; Wahid *et al.* 2007). For example, heat stress imposition may delay or completely inhibit seed germination. At later developmental stages, high temperatures can cause a significant number of injuries, including premature leaf senescence and abscission, sunburns, shoot and root growth inhibition and fruit discoloration and damage. Concerning the high variability of morphological responses, it appears that the effect of heat stress varies with plant species and developmental stages (reviewed by Wahid *et al.* 2007). Reproductive processes are also highly susceptible to high temperatures in most plants, specifically anthesis and grain filling, resulting in reduced yield.

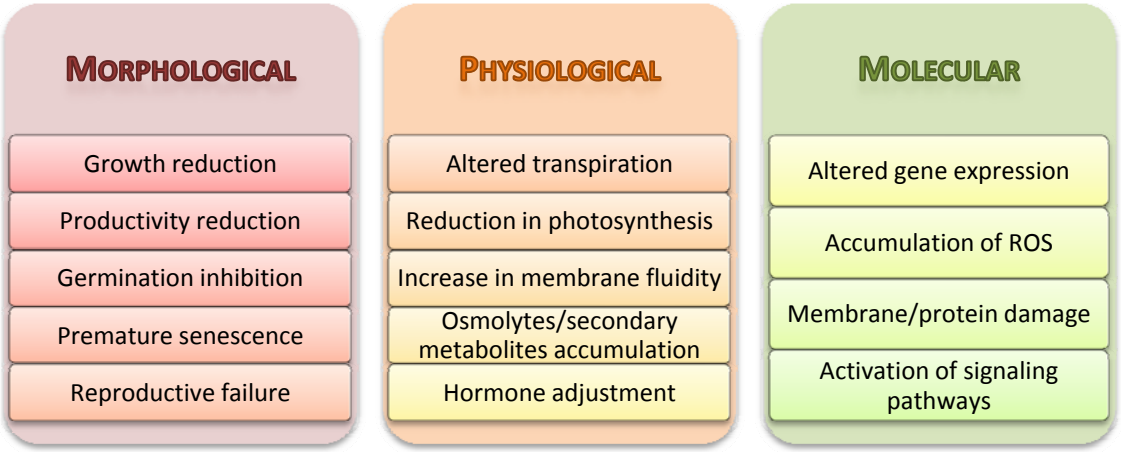

Figure 1.2

Overview of plant morphological, physiological and molecular responses induced by heat stress.

Physiological responses

High temperatures result into enhanced transpiration and resultant water loss, leading to the disruption of several physiological processes (reviewed by Madhava Rao *et al.* 2006; Wahid *et al.* 2007). Heat stress also causes reduced efficiency of photosynthesis, impaired translocation of assimilates and loss of carbon gain. In turn, these factors merge to cause altered phenology, reproductive failure and accelerated senescence. High temperatures lead to enhanced fluidity of membranes through either shifting lipid properties or protein denaturation, thereby promoting the failure of membrane processes such as photosynthesis and respiration (reviewed by Sung *et al.* 2003; Wahid *et al.* 2007). Several approaches, like mutational analysis, transgenic and physiological studies established the importance of proper membrane fluidity in temperature tolerance. For example, mutants with increased lipid saturation in membrane composition displayed stronger tolerance to high temperatures (Murakami *et al.* 2000; Alfonso *et al.* 2001).

Increased temperatures also modulate the levels of compatible osmolytes, secondary metabolites and hormones (Wahid *et al.* 2007). Compatible solutes (*e.g.*, sugars, polyols, proline) are important for maintenance of cell turgor by stimulating water uptake (reviewed by Wang *et al.* 2003). These organic compounds of low molecular weight can also act as ROS scavengers or chemical stabilizers of membranes and/or proteins. Secondary metabolites like phenolics or carotenoids are widely

known for their protective effects. The accumulation of diverse types of secondary metabolites under heat stress has been related to different effects, such as the prevention of oxidative damage to the membrane lipids or protection of the photosynthetic apparatus (reviewed by Wahid *et al.* 2007). Hormones play an important role as secondary signals involved in signal transduction that can trigger the molecular events leading to many physiological responses (Kaur and Gupta 2005). Abscisic acid (ABA), ethylene and salicylic acid (SA) are some examples of plant hormones which have been shown to confer enhanced thermotolerance (reviewed by Kotak *et al.* 2007; Wahid *et al.* 2007). The accumulation of compatible osmolytes and secondary metabolites, as well as the regulation of hormone production caused by heat stress, has been considered to be adaptive mechanisms contributing to enhance stress tolerance. However, the specific roles they play in enhancing thermotolerance still demands further research (Wahid *et al.* 2007).

Molecular responses

Immediately after exposure to increased temperatures and signal perception, alterations at the molecular level result in the activation of gene expression and accumulation of transcripts (reviewed by Wahid *et al.* 2007). The synthesis and accumulation of HSPs and other stress-related proteins represent an important and ubiquitous adaptive strategy to heat stress. The so-well characterized HSPs have a particular key role in cellular protein quality control and in stabilizing proteins and membranes under abiotic stress (Kotak *et al.* 2007; Liberek *et al.* 2008). Ranging in molecular weight from 10 to 200 kDa, heat stress-induced HSPs play an important role in maintaining proteins in their functional conformation, preventing aggregation of non-native proteins, refolding denatured proteins and removing non-functional polypeptides arising from misfolding, denaturation or aggregation (Wang *et al.* 2004). They also provide protection of functional sites, assist in protein translocation and are involved in signal transduction during heat stress leading to transcriptional activation of other HSPs (reviewed by Wahid *et al.* 2007). Indeed, the protective effects of HSPs can be attributed to the network of chaperone machinery, in which many chaperones act in concert. The tolerance to heat stress provided by HSPs results in improvement of physiological phenomena, such as photosynthesis, assimilate partitioning, water and

nutrient use efficiency and membrane stability. Among the other stress-induced proteins, ubiquitin, late embryogenesis proteins (LEA) and dehydrins have also important roles in stress defense (reviewed by Wahid *et al.* 2007). These proteins have been suggested to function as molecular chaperones under heat stress, assisting in protection of essential and membrane proteins from the adverse effects of oxidative stress and dehydration. Decreases in transcripts related to programmed cell death (PCD), basic metabolism and biotic stress responses have recently been found to be equally important for adaptation to high temperature (Larkindale and Vierling 2008).

At high temperatures, the generation of reactive oxygen species (ROS) constitutes another major plant response caused by the disruption of cellular homeostasis and uncoupling of metabolic processes (reviewed by Suzuki and Mittler 2006). This enhanced production of ROS leads to the induction of the antioxidant system (reviewed by Dat *et al.* 2000). The levels of ROS-scavenging enzymes in coordination with antioxidant compounds are responsible for reducing ROS levels and oxidative damage, providing enhanced tolerance to heat stress. Therefore, an inadequate control of ROS levels can lead to phytotoxicity, culminating in PCD or ultimately in necrosis. However, the accumulation of relatively low levels of ROS is useful for the activation of stress-response pathways by signal transduction (reviewed by Suzuki and Mittler 2006; Van Breusegem and Dat 2006). During the course of evolution, it seems that plants were able to achieve a higher degree of control over ROS toxicity and use these molecules as signaling components. It is the level of signaling molecules and/or ROS that results on the activation of PCD, which is responsible for development and maintenance of plants by removing redundant, misplaced or damaged cells.

Plant adaptation to heat stress also involves protein degradation executed by proteases, including enzymes in the ubiquitin-proteasome pathway and other proteolytic proteases, which have been found to be up-regulated by high temperature. This stimulation of protein degradation, which was suggested to be relatively unspecific, could affect several enzymes (Huang and Xu 2008).

It is the tight regulation of the several adaptive mechanisms referred above that enables plants to thrive under heat stress.

TEMPERATURE PERCEPTION AND SIGNAL TRANSDUCTION

Before turning on the molecular responses, plants have to perceive the stress whenever it occurs and transduce the information through a signaling pathway (reviewed by Penfield 2008). Despite the recent progress on understanding the regulation of the heat stress response, how the temperature signal is perceived and is further relayed remains largely unknown. Modifications of membrane fluidity (both by low and high temperatures) have been suggested to alter gene expression (reviewed by Sung *et al.* 2003). Cellular membranes are now considered to be the initial sites of temperature perception. Indeed, one of the immediate effects of temperature stress is the adjustment of membrane saturated/unsaturated fatty acid levels, which could be considered as a damage and/or represent a site of perception (Wahid *et al.* 2007).

Several signaling pathways have been implicated in the response leading to thermotolerance (Figure 1.3) (reviewed by Kotak *et al.* 2007). The network represented by the multiple heat shock transcription factors (HSFs) is clearly of major importance by their regulator effect on the transcription of *HSP* genes. Also, a number of chemical signals, such as Ca^{2+}, ROS, ethylene and plant hormones, has been identified to induce signaling cascades (reviewed by Wahid *et al.* 2007). In addition, evidence of cross-talking between different signaling cascades has been suggested (*e.g.*, heat and oxidative stress signaling).

Special attention has been given to the particular role of calcium (reviewed by Sung *et al.* 2003; Wahid *et al.* 2007). An increase in membrane fluidity after heat stress has been shown to lead to a raise in cytosolic Ca^{2+} content and cytoskeletal reorganization. It has been proposed that cytosolic Ca^{2+} might be involved in transducing heat-induced signals to mitogen activated and calcium dependent protein kinases (MAPK and CDPK, respectively). These kinases are well-known components of signal transduction pathways in plants, which ultimately lead to nuclear production of antioxidants and compatible osmolytes. The role of ROS in signaling and activating the production of antioxidants and compatible osmolytes has been established (reviewed by Wahid *et al.* 2007). Moreover, ROS signaling has been shown to activate the

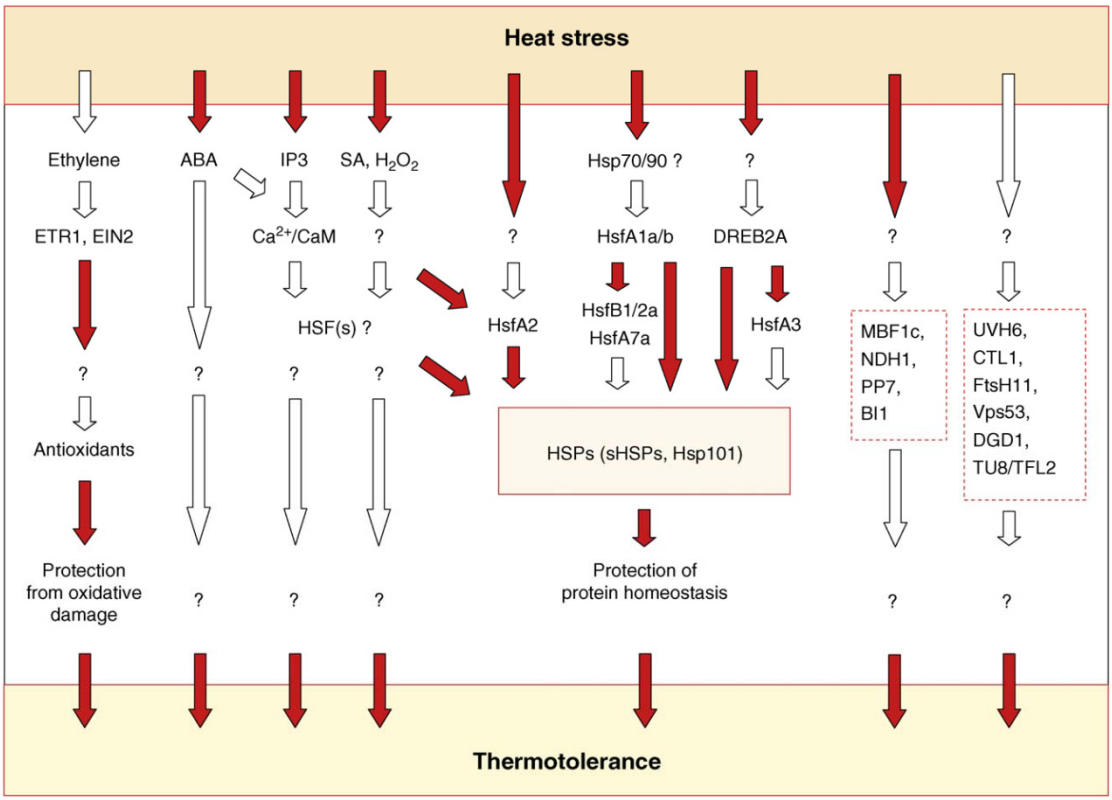

Figure 1.3

Overview of the multiple signaling pathways and factors implicated in the heat stress response. The heat shock transcription factors (HSFs) are one of the main components of the network, mediating the expression of protective proteins (HSPs) and thereby leading to thermotolerance. Red arrows point to connections with experimental evidence, while open arrows indicate hypothesized connections. Question marks illustrate unidentified components of the corresponding signal transduction pathways. Boxes with red dotted lines represent a collection of gene products that are known to affect thermotolerance, but whose particular functions in the network are still unknown (Kotak *et al.* 2007).

temperature defense pathway controlling HSPs expression (reviewed by Suzuki and Mittler 2006). For example, a burst of H_2O_2 caused by short exposures to high temperature has been correlated with the induction of heat stress-responsive genes through direct activation of HSFs (reviewed by Kotak *et al.* 2007). In addition to the referred signaling pathways, other specific groups of molecules with potential signaling activity (SA, ABA and ACC) have been suggested to indirectly enhance tolerance to heat stress by reducing oxidative injury or inducing HSPs synthesis (Larkindale and Huang 2004). An adequate response in the signaling and gene activation pathways is vital for maintenance of cellular homeostasis and protection of proteins and

membranes, thereby preventing cell death (Vinocur and Altman 2005; Bohnert *et al.* 2006).

TRANSCRIPTIONAL REGULATION

The regulation of transcription is assumed to play a fundamental role in the response and adaptation to heat stress. A conserved target sequence (heat shock element, HSE), found in the promoter of many heat-inducible genes, was found to serve as the binding site for HSFs (Kotak *et al.* 2007). Following their activation by oligomerization in the cytosol, HSFs are translocated to the nucleus where they bind to HSE (Baniwal *et al.* 2004). HSFs are known to be the key regulatory proteins that mediate the expression of genes induced both by heat stress and several chemical stressors (Von Koskull-Döring *et al.* 2007).

A high number of HSFs are found in plants, which can be distributed in three major classes according to their structure (HSFA, HSFB and HSFC). While class A HSFs appears to be the major transcription factors responsible for heat-induced activation of heat shock-associated genes, the function of class B and C is less conclusive. One of the best well characterized HSF system resulted from genome-wide transcriptome studies of wild-type and HSF mutant lines in *Arabidopsis thaliana* (reviewed by Kotak *et al.* 2007; Von Koskull-Döring *et al.* 2007). Analysis of *Arabidopsis* knockout mutant lines of *HSFA1a*, *HSFA1b* and *HSFA2* suggested that HSFA1a and HSFA1b are essential for the initial phase of heat-responsive gene expression, and that HSFA2 regulates expression under prolonged stress and during recovery (Figure 1.4). Interestingly, HSFA2 was reported to be also induced by high light and H_2O_2. Also, the *Arabidopsis* transcriptome analysis performed using AtGenExpress microarray data during heat stress suggests that *HSFA2* is strongly induced in all tissues (Von Koskull-Döring *et al.* 2007). To a certain extent, this was also verified for *HSFA7a*, *HSFA7b*, *HSFB1*, *HSFB2a* and *HSFB2b* transcripts. Besides the regulation of heat stress response, the transcript profiles of HSFs during development and during other abiotic and biotic stress responses suggests their involvement in other signaling cascades (Figure 1.4) (Von Koskull-Döring *et al.* 2007). Examples of the involvement of HSFs in the regulation of

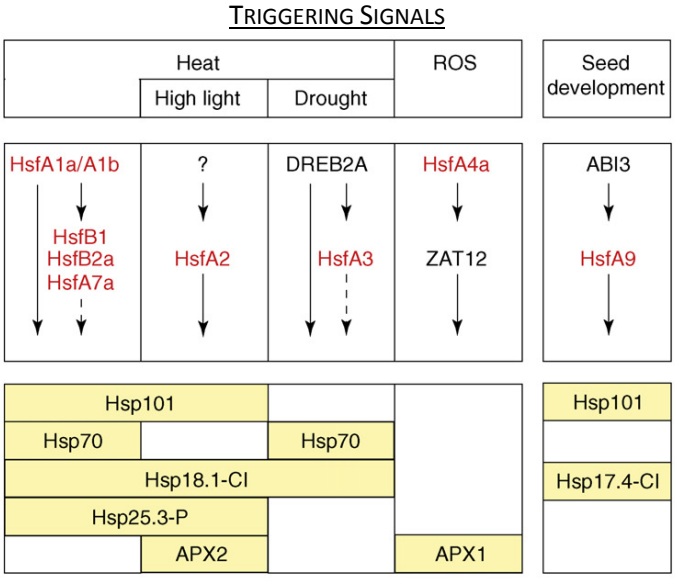

Figure 1.4

Model representation of the role of HSFs in different signal transduction pathways. The model was proposed based on functional analysis of *Arabidopsis* mutant lines of *HSFA1a* and *HSFA1b*, *HSFA2*, *HSFA4a*, *HSFA9*, *DREB2A*, *APX1* and *ABI3*. The different triggering signals lead to the activation of distinct transcriptional pathways including HSFs (red), which can induce directly or indirectly the expression of target genes (yellow boxes). HSFs can be either co-regulated with the target genes or can act as regulatory modulators (dotted lines) (Von Koskull-Döring *et al.* 2007).

antioxidant enzymes and dehydration-responsive genes, in ROS perception or during seed development have been reported. Although the recent knowledge in the regulation of gene expression during heat stress response, the understanding of HSF network and their association with other signaling pathways and transcription factors is still far from complete.

FUNCTIONAL GENOMICS AND GENETIC IMPROVEMENT FOR HEAT TOLERANCE

A key to progress towards improvement of plant tolerance has been the understanding of stress adaptive mechanisms at the whole plant, cellular and molecular levels (reviewed by Wang *et al.* 2003; Vinocur and Altman 2005; Bhatnagar-Mathur *et al.* 2008). Functional genomics provides tools for revealing the complex networks of stress perception and signal transduction that lead to the multiple defensive responses to abiotic stress (reviewed by Langridge *et al.* 2006; Sreenivasulu *et al.* 2007; Vij and Tyagi 2007). The recent advances on genomics, proteomics and

metabolomics, together with all the potentialities of bioinformatics, now enable to easily evaluate the global profiles of transcripts, proteins and metabolites of stressed tissues. High-throughput studies for unravel the function of stress-responsive genes also include the use of mutant and transgenic plants (reviewed by Vij and Tyagi 2007).

Genetic engineering strategies for improving abiotic stress tolerance currently rely on the expression of specific-related genes (reviewed by Bhatnagar-Mathur *et al.* 2008). Avoidance mechanisms by modification of morphology and anatomy, although being a highly developed defense system to temperature stress, may not be of much importance in immediate improvement of plant tolerance. Plant modification for enhanced tolerance to stress have been mostly based on the manipulation of three different categories of genes: a) those involved in signaling transduction and transcriptional regulation, b) those that function in membrane and protein protection and c) those related to water and ion uptake and transport (reviewed by Wang *et al.* 2003). Several comprehensive reviews on effective engineering strategies leading to greater heat stress tolerance have been published (Iba 2002; Sreenivasulu *et al.* 2007; Vij and Tyagi 2007; Bhatnagar-Mathur *et al.* 2008; Singh and Grover 2008). It is important to notice that genetic engineering of genes involved in thermotolerance may be beneficial in the production of heat-tolerant plants, as well as in the understanding of its functional roles. High temperature tolerance has been achieved by overexpression of HSP genes (or indirectly by elevating the levels of HSF proteins), by increasing levels of osmolytes and cell detoxification enzymes and by changing membrane fluidity (reviewed by Singh and Grover 2008). All of these approaches have induced thermotolerance in transgenic plants by acting at the level of protein metabolism through minimizing the accumulation of damaged proteins (Singh and Grover 2008). HSPs are directly implicated in the regulation of denatured/aggregated protein levels through their chaperone activity (reviewed by Wang *et al.* 2004; Kotak *et al.* 2007). Increasing osmolytes and detoxifying enzymes and altering membrane fluidity have been suggested to create a more reductive and energy-rich cellular environment, thereby protecting active proteins from damage (Singh and Grover 2008). A large number of genetically engineered genes have been extensively discussed by Singh and Grover (2008) and some representative examples are shown in Table 1.1.

Table 1.1 Some representative examples of plant transgenes raised for heat tolerance (adapted from Singh and Grover 2008).

Gene	Protein (cellular function)	Source → Trans host	Comments	Reference
Using HSFs and HSPs				
HSF1	HSF1 (transcription factor)	*Arabidopsis thaliana* → *Arabidopsis thaliana*	Transformants exhibited thermotolerance and also constitutive expression of the *HSP* genes at normal temperature.	Lee *et al.* (1995)
HSFA2	HSFA2 (transcription factor)	*Arabidopsis thaliana* → *Arabidopsis thaliana*	The mutants displayed reduced basal and acquired thermotolerance as well as reduced oxidative stress tolerance, while the overexpression lines displayed increased tolerance.	Li *et al.* (2005)
sHSP	MT-sHSP (chaperone)	*Solanum lycopersicon* → *Nicotiana tabacum*	Plants overexpressing the *MT-sHSP* gene exhibited thermotolerance, while antisense plants in which the expression of the gene was suppressed exhibited susceptibility.	Sanmiya *et al.* (2004)
HSP101	HSP101 (chaperone)	*Arabidopsis thaliana* → *Arabidopsis thaliana*	Transformants constitutively expressing *HSP101* tolerated sudden shifts to extreme temperatures better than the controls.	Queitsch *et al.* (2000)
Using proteins involved in ROS scavenging system				
APX1	Ascorbate peroxidase (H_2O_2 detoxification)	*Hordeum vulgare* → *Arabidopsis thaliana*	Transgenic plants were significantly more tolerant to heat stress.	Shi *et al.* (2001)
Using proteins involved in osmolyte synthesis				
BADH	Betaine aldehyde dehydrogenase (glycinebetaine synthesis)	*Spinacia oleracea* → *Nicotiana tabacum*	Transgenic plants accumulated glycinebetaine, mainly in chloroplasts, which resulted in enhanced tolerance to high temperature stress during growth of young seedlings.	Yang *et al.* (2005)
codA	Choline oxidase A (glycinebetaine synthesis)	*Arthrobacter globiformis* → *Arabidopsis thaliana*	Transformants showed tolerance to high temperature during seed imbibition and germination.	Alia *et al.* (1998)
Using proteins involved in lipid metabolism associated with membranes				
FAD7	ω-3-fatty acid desaturase (causes reduction of trienoic fatty acids and hexadecatrienoic acid)	*Arabidopsis thaliana* → *Nicotiana tabacum*	Transformants showing silencing of the gene were better able to acclimate to higher temperature.	Murakami *et al.* (2000)
		Arabidopsis thaliana → *Oryza sativa*	Transformants showing silencing of the gene showed better growth, higher chlorophyll content and photochemical efficiency.	Sohn and Back (2007)

Although the use of transformation technology may have a significant impact in the development of transgenic plants with improved stress tolerance, there are only a limited number of successful examples (reviewed by Wang *et al.* 2003; Bhatnagar-Mathur *et al.* 2008). Nonetheless, based on a comprehensive understanding of the mechanism of stress tolerance, genetic strategies have been used for the manipulation of heat stress responses using modern molecular breeding and transgenic approaches (reviewed by Sung *et al.* 2003; Wahid *et al.* 2007). In addition to genetic approaches, different methods have been successfully used to induce heat tolerance in several

plant species. Traditional breeding of existing high-yielding cultivars has been reported, as well as the imposition of preconditioning heat treatment or application at seed and whole plant level of exogenous osmoprotectants (reviewed by Wahid *et al.* 2007).

Considering the recent advances on stress-specific gene participation in plant adaptation to heat stress, genetic engineering approaches for improvement of plant heat tolerance will be more widely used in a nearly future. Future advances on understanding the complex heat stress-responsive networks and their associated genes are expected to expand the number of candidate targets for plant manipulation towards the production of highly thermotolerant plants.

THE ROLE OF MODEL SPECIES

Functional genomics studies have been facilitated by the rising number of tools and resources in traditional models (Cushman and Bohnert 2000; Bohnert *et al.* 2006; Langridge *et al.* 2006). *Arabidopsis* has been an excellent model species for studying the plant stress responses and for the identification of target genes for biotechnology applications (reviewed by Zhang *et al.* 2004; Bevan and Walsh 2005). The knowledge gained from *Arabidopsis* research may enable the understanding of the function of heat stress-relevant genes and therefore contribute to the production of commercial plant varieties with improved performance. Another excellent parallel approach has been to perform comparisons between model species and natural stress-tolerant relatives (Bohnert *et al.* 2006). Plant stress biology may profit from evaluation of transcript profiling from contrasting genotypes with respect to stress tolerance, allowing the identification of potential candidates for mediating stress tolerance. Regarding heat tolerance, *Populus euphratica*, a tree species naturally adapted to extreme conditions (including high temperatures), might be a good studying model system. Besides its natural tolerance, studies on *Populus* spp. are facilitated by its close phylogenetic relationship with *Arabidopsis* and due to the recent development of genomic and molecular biology resources for *Populus* genus (Gu and Pei 2005; Jansson and Douglas 2007).

AIM OF THE THESIS

The research presented in this work aimed to contribute for a better understanding of the physiological and molecular mechanisms responsible for plant tolerance to high temperatures. For our purpose, the model systems *P. euphratica* and *A. thaliana* provided special interest due to the recent increase of sequence information and development of genomic resources and tools. Taking into account *P. euphratica* natural tolerance to extreme temperatures, particular emphasis was devoted to the physiological characterization of poplar cell suspensions subjected to different temperatures. The work performed in *A. thaliana* pretended to provide new insights in the regulatory networks that control thermotolerance in plants. By making use of the expression data acquired in publicly available microarray experiments, the studies concerned primarily the identification of novel thermotolerance determinants. Further functional characterization of a selected determinant gene was supported by the phenotypic analysis of loss-of-function mutants and by the use of several tools and resources specifically created for *Arabidopsis* functional analysis.

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Chapter 2

Understanding thermotolerance of *Populus euphratica* suspended cells

Silva-Correia, J., Azevedo, H., Pais, M.S., Tavares, R.M. and Lino-Neto, T. (2009) Heat stress thermotolerance of *Populus euphratica* suspended cells. Plant Cell, Tissue and Organ Culture *(submitted)*

CONTENTS

Understanding thermotolerance of *Populus euphratica* suspended cells

ABSTRACT

Extreme temperatures are one of the most serious environmental threats faced by plants all over the world. Temperature fluctuations lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity. A comprehensive understanding of physiological responses of plants to extreme temperatures and the mechanisms by which they can tolerate thermal stress is essential for future strategies to improve plant thermotolerance. Advantage can be taken by using extremely adapted model plants, like the stress-tolerant tree species, *Populus euphratica* Oliv., which is naturally found under harsh conditions such as extreme temperatures (-45°C to +54°C), high soil salinity and drought. Its natural tolerance along with a relatively small genome and easy manipulation *in vitro* made it an excellent model to the study of mechanisms responsible for thermotolerance.

In the present study, the physiological characterization of heterotrophic cell suspensions of *P. euphratica* was attempted by subjecting cells to different temperatures. The evaluation of cell viability and dry weight upon thermal shock revealed that 45°C corresponds to the sub-lethal temperature for *P. euphratica* cells heat-shocked for 20 min. In these conditions, suspended cell culture was able to recover, after a period of decline in cell viability due to the occurrence of cell death. Furthermore, heat-associated events as PCD and production of ROS may not be implicated in the process of induction of the observed cell death.

KEYWORDS Abiotic stress · Necrosis · *Populus euphratica* · Programmed cell death · Reactive oxygen species · Suspension cell cultures · Thermotolerance

ABBREVIATIONS BAP: 6-**b**enzyl**a**mino**p**urine · Carboxy-H_2DCFDA: 5-(and-6)-**carboxy**-2',7'-**di**chlorodihydro**f**luorescein **dia**cetate · DW: **d**ry **w**eight · EST: **e**xpressed **s**equence **t**ag · HBSS/Ca/Mg: **H**ank's **b**alanced **s**alt **s**olution with calcium and magnesium · HSP: **h**eat **s**hock **p**rotein · MS: **M**urashige and **S**koog · NAA: 1-**n**aphthalene**a**cetic **a**cid · PCD: **p**rogrammed **c**ell **d**eath · ROS: **r**eactive **o**xygen **s**pecies · TUNEL: **t**erminal deoxynucleotidyl transferase d**U**TP **n**ick **e**nd **l**abeling

INTRODUCTION

In natural environment, plants often grow under unfavourable conditions that adversely affect plant development and productivity. Particularly, exposure of plants to adverse temperatures can significantly affect many essential metabolic processes and disrupt an extensive range of cellular components. Three distinct types of this kind of stress can be found in nature: low temperatures above 0°C (chilling temperatures), temperatures below 0°C (freezing temperatures) and high temperatures, all responsible for a variety of responses. When facing extreme temperatures, plants experience stresses of variable severity that depend on the intensity and extent of stress and the velocity of temperature variation. As sessile organisms, plants have developed several metabolic responses that minimize injures caused by the constant exposure of plants to daily temperature fluctuations and other abiotic factors (Wang *et al.* 2003). The level of tolerance a given plant displays depend on the plant, tissue or cell type considered (Sung *et al.* 2003).

The continuous progress on the understanding of plant tolerance mechanisms to abiotic stresses has been allowing the development of more effective engineering strategies leading to enhanced stress tolerance. The extensive list of targets for genetic engineering include, among others, osmoprotectants, reactive oxygen scavengers, heat shock proteins (HSPs) and other stress proteins, signaling components and transcription factors (Bhatnagar-Mathur *et al.* 2008). As accomplished for other abiotic stresses, plant transformation with genes enhancing thermal tolerance has been successfully achieved (Iba 2002; Sung *et al.* 2003). However, even thought a huge progress in the understanding of the temperature stress mechanisms in plants has been brought in the recent years, the function of several genes is still unknown. A strategy for gene function assignment has been the utilization of plant model species that are naturally adapted to survive in extreme environments. In these species, regulatory mechanisms enabling their survival on such adverse conditions are still active and could be used for revealing a set of genes important for plant stress tolerance (Cushman and Bohnert 2000; Vinocur and Altman 2005).

Unique attributes made the natural tolerant species *Populus euphratica* Oliv. (Figure 2.1) a promising candidate. This model system is a large deciduous tree which

belongs to the family Salicaceae, order Salicacae, genus *Populus* and section Turanga Bga. *P. euphratica* can present polymorphic leaves on the same tree and even in the same branch, although the most typical are from the lanceolate-linear type. Usually, poplar proliferates by seed germination but classical rooting of cuttings is used as well (Taylor 2002). The advantages of using the members of the genus *Populus* as genomic systems for tree molecular biology are innumerous and have been reviewed extensively (Brunner *et al.* 2004). A small genome of approximately 480 Mbp and the

Figure 2.1

Populus euphratica phenological features of trees growing in their natural desert habitat (Negev desert, Israel), depicting wide trunks and large healthy crowns (Brosché *et al.* 2005).

facility of being genetically transformed, regenerated and vegetatively propagated are the most crucial ones. Another important feature is its relatively close phylogenetic relationship to *Arabidopsis* in the Eurosid clade of Eudicotyledonous plants (Figure 2.2). The extensive knowledge of *Arabidopsis* and the recently developed genomic and molecular biology resources for the *Populus* genus promotes the identification of conservative processes in plants by comparative functional and genomic studies (Jansson and Douglas 2007).

When compared to the other genus species, *P. euphratica* shows a remarkable survival and biomass production under diverse environmental stresses, particularly to high soil salinity, extreme temperature and drought (Gu and Pei 2005). These adverse conditions are typically found in the semi-arid areas over the wide longitudinal stretch from China to Spain and from Kenya in the south to Kazakhstan, where *P. euphratica* is

Figure 2.2

Angiosperm phylogeny showing the Eurosid clade containing *Populus* and *Arabidopsis* (red arrows), relative to other species with significant sequence information (highlighted in color). Data and images were adapted from P. F. Stevens (2001 onwards), Angiosperm Phylogeny Web site: **http://www.mobot.org/MOBOT/research/APweb/** (Version 9, June 2008).

naturally distributed (Wang *et al.* 1996). Continuous efforts had been done in order to understand poplar tolerance to salinity and drought stresses (Ma *et al.* 1997;

Watanabe *et al.* 2000; Ma *et al.* 2002; Gu *et al.* 2004*a;* Gu *et al.* 2004*b;* Ottow *et al.* 2005; Bogeat-Triboulot *et al.* 2007; Yang *et al.* 2007). Enhanced salt tolerance has been recently achieved in transgenic tobacco containing a stress responsive zinc-finger protein gene from *P. euphratica* (Wang *et al.* 2008). Considering temperature adaptation, *P. euphratica* can survive environments with annual extreme temperatures ranging from -45°C to +54°C (Gu and Pei 2005). However, although being known as a natural temperature tolerant plant, the reason why and how *P. euphratica* can survive such extreme temperatures is not so well investigated (Brosché *et al.* 2005; Ferreira *et al.* 2006). Brosché *et al.* (2005) reported an elegant approach to identify genes involved in abiotic stress responses by comparing expression profiles of poplar trees in controlled environment, stress-imposed and natural conditions. Apparently, the natural adaptation of *P. euphratica* to saline semi-arid environments might be due to specific regulation of gene expression rather than the presence of particular genes by itself (Brosché *et al.* 2005). Another effort for the understanding of *P. euphratica* thermotolerance comprised an extensive analysis of the protein accumulation profiles after imposition of a moderate heat stress (Ferreira *et al.* 2006). In order to mimic the day-night temperature fluctuations, hydroponic cultures of *P. euphratica* were submitted to light/dark cycles of 42/37°C for three days. Short- and long-term regulated proteins were identified, which were mainly involved in lipid metabolism, cytoskeleton restructure, sulphate assimilation, amino acid biogenesis and nuclear translocation. It was also suggested that *P. euphratica* cultures reached photostasis by photosynthesis and carbon metabolism adjustments (Ferreira *et al.* 2006).

Several specific features highlight the attractiveness of *in vitro* cultured cells as a system to study stressed-cell responses. Cell cultures rapidly generate a great amount of cell material with reduced cellular complexity. More importantly, studies carried out in this kind of tissue culture allow to completely control the homogeny of cell response and environmental parameters. Cell cultures also allow a number of quantitative studies in which compounds can be simply added or removed from the medium and cell aliquots can be easily harvested (McCabe and Leaver 2000). A number of protocols to establish stable cell suspensions for studying plant stress responses has been reported from several plant species, including *Arabidopsis*, cucumber, *Zinnia*, soybean and others as reviewed by McCabe and Leaver (2000). In

particular, the use of cell suspension cultures for the evaluation of heat stress responses has been successfully accomplished for several plant species. Some representative examples are tomato (Scharf and Nover 1982), pear (Wu and Wallner 1984), carrot (McCabe *et al.* 1997), *Arabidopsis* (McCabe and Leaver 2000; Lim *et al.* 2006) and tobacco (Vacca *et al.* 2004; Burbridge *et al.* 2007). The general focus of these studies was the evaluation of plant tolerance ability, analysis of gene expression profiles, detection of programmed cell death (PCD) and identification of photosynthetic and mitochondrial metabolism alterations. In the well-documented *Arabidopsis* and tobacco species, suspension cultures can easily tolerate heat stresses lower than 45°C for 10 and 20 min, respectively (McCabe and Leaver 2000; Burbridge *et al.* 2007). When exposed to temperatures above 45°C, both model systems started to display cell death, which was maximally triggered by PCD in 55°C heat-stressed cells. A similar result was reported for tobacco cultured cells which had experienced heat stress (55°C) during 10 min (Vacca *et al.* 2004). In these experiments, studies were essentially directed to the mechanism of PCD induction by heat and focused mainly in cell viability effects, cell morphology, reactive oxygen species (ROS) production and antioxidants levels. The strategy of using cell suspension culture will be specifically advantageous when studying thermotolerance mechanisms among trees. The particular use of *P. euphratica* suspension cultures had already been reported in saline and osmotic tolerance studies (Ma *et al.* 2002; Gu *et al.* 2004*b*). However, as far as we know, no studies concerning thermotolerance in this system were performed up till now.

Considering the outlined questions and the reduced information in thermotolerance of the natural model *P. euphratica*, the earliest aim of the present work was to evaluate the heat stress tolerance ability of the corresponding cell cultures. Suspended cells were subjected to a range of temperatures and cell viability and biomass content were determined at regular intervals. In order to elucidate the physiological mechanisms that enable this species to cope with adverse temperatures, particular emphasis was then given to the occurrence of heat-associated events in heat-stressed cells, like PCD and overexpression of ROS.

MATERIAL AND METHODS

Cell suspensions and growth conditions

Populus euphratica calli (Gu *et al.* 1999) were kindly provided by R. Gu (Laboratory of Plant Biotechnology, ICAT, Portugal) and were used for the induction of cell suspension cultures. Initiation of cell cultures was performed using 4-week-old *callus* by transferring 1-2 g of tissue to 70 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 0.5 $mg.l^{-1}$ 1-naphthaleneacetic acid (NAA), 0.25 $mg.l^{-1}$ 6-benzylaminopurine (BAP) and 2.5% (w/v) sucrose, at pH 5.5. The 70 ml cultures were maintained in 250 ml flasks, at 25°C, with continuous shaking (125 rpm) in the dark. Cell suspension subcultures were performed every 12 days by transferring 10 ml of cell culture into 60 ml of fresh medium.

Dry weight determination

Cell growth was monitored by determination of dry weight for estimation of cell specific growth rates (μ). Aliquots of 3 ml of suspended cells were harvested and filtered using a pre-weighted filter paper (Whatman). Dry weight was determined after oven drying at 60°C for 24 h. This procedure was repeated every 2 days, over a total period of 16 days. Biomass values were transformed into their natural logarithm for specific growth rate calculation (μ).

Temperature stress treatments

At day 6 of growth, midlog phase cell suspension flasks were immersed into a water bath at different temperatures (5 to 75°C), for 20 min, with continuous shaking. After temperature stress, the cells were transferred into another water bath, at 25°C for 10 min, for recovering the usual growth temperature. Suspension cultures were then returned to the previous incubation conditions (25°C, with 125 rpm continuous shaking, in the dark). At regular intervals, cell aliquots were collected for cell viability analysis and dry weight determination.

Determination of cell viability and morphology

Cell viability of suspended cells was measured using trypan blue exclusion method. Aliquots of 150 µl of cell culture were gently mixed with identical volume of 0.4% (w/v) trypan blue (Sigma-Aldrich) and incubated in the dark for 10 min. A sample of 50 µl was observed under a Leica ATC2000 light microscope and the number of viable (unstained) and dead cells (stained) were determined. Cell viability is referred in relation to the total number of the cells observed. When cell death was observed, cell viability values were transformed into their natural logarithm for specific death rate determination. Changes of cell morphology were observed with a Leitz Laborlux S epifluorescence microscope (Leitz) under phase contrast. Images were recorded with a 3CCD color video camera (Sony, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma P) and software for image management and archiving (AxioVision Version 3.0, Carl Zeiss Vision, GmbH).

Programmed cell death evaluation

Midlog phase cell suspensions were heat stressed at 45 or 50°C, under the same experimental conditions referred above. Programmed cell death was evaluated before and immediately after heat stress, as well as during recovery period at 25°C, after 1, 4, 6 and 8 h of heat treatment. PCD was revealed by TUNEL assay, as originally described by Gravieli *et al.* (1992), through the detection of DNA fragmentation. Terminal deoxynucleotidyl transferase (TdT) polymerizes fluorescein-conjugated nucleotides to free 3'-OH termini, allowing their detection by fluorescence microscopy. The reactions were performed using the In Situ *Cell Death Detection Kit – Fluorescein* (Roche Applied Science), according to supplier instructions and as described by Sgonc *et al.* (1994). Briefly, aliquots of 3 ml of suspended cells were washed once with PBS [8 $g.l^{-1}$ NaCl; 0.2 $g.l^{-1}$ KCl; 1.44 $g.l^{-1}$ Na_2HPO_4; 0.24 $g.l^{-1}$ KH_2PO_4] and subsequently mixed with a similar volume of freshly prepared fixation solution [4% (v/v) paraformaldehyde in PBS, pH 7.4]. Fixation occurred for 1 h at 20°C and cells were subsequently gently washed in PBS and incubated for 10 min at 20°C in blocking solution [3% (v/v) H_2O_2 in methanol]. After washing in PBS, cells were incubated on ice for 2 min in permeabilisation solution [0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate]. After two additional washings in PBS, 50 µl of TUNEL solution (5 µl of Enzyme Solution mixed

with 45 µl of Label Solution, Roche Applied Science) were added to approximately equal volume of cells. Incubation was performed for 60 min at 37°C in the dark. Finally, labelled cells were gently washed three times with PBS and mounted in a glass slide. Positive and negative controls were performed as indicated by supplier instructions. The samples were visualized using a Leitz Laborlux S epifluorescence microscope (Leitz) under a 50W mercury lamp with an excitation wavelength of 495 nm and a detection wavelength within the range of 515-565 nm. Images were recorded as described in "Determination of Cell Viability and Morphology".

Detection of ROS production

Reactive oxygen species (ROS) production was evaluated in heat (45 or 50°C) stressed cells, under the same experimental conditions as referred above. Assays were performed before and immediately after heat stress, as well as during recovery period at 25°C (2, 6, 12 and 29 h after heat treatment). Non-specific ROS were detected by *Image-iT^{TM} LIVE Green Reactive Oxygen Species Detection Kit* (Molecular Probes), which is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA). This non-fluorescent compound permeates live cells and is deacetylated by non-specific intracellular esterases. The obtained fluorescein product is oxidized in the presence of non-specific ROS and emits bright green fluorescence. The assay was performed according to supplier instructions. Briefly, 5 ml aliquots of suspended cells were gently washed with the same volume of warmed Hank's balanced salt solution with calcium and magnesium (HBSS/Ca/Mg, Gibco). Cells were first mounted in a glass slide and those adhering to the coverslip were directly labelled by applying 25 µM carboxy-H_2DCFDA working solution. Stained cells were incubated for 30 min at 37°C, in the dark. Finally, the coverslip was gently washed three times with warmed HBSS/Ca/Mg solution and cells were immediately imaged. As the oxidized compound has an excitation/emission maxima of approximately 495/529 nm, positively reactive cells were detected under a Leitz Laborlux S epifluorescence microscope (Leitz). Samples were also stained with Hoechst 33342 (350/461 nm), a blue fluorescent cell-permeant nucleic acid stain supplied with the kit. For achieving this, 0.25 µl of Hoechst 33342 stain (1 mM, Molecular Probes) was added to the

carboxy-H_2DCFDA staining solution during the last 5 min of the incubation step. Images were recorded as described in “Determination of Cell Viability and Morphology”.

RESULTS AND DISCUSSION

Physiological characterization of cell suspensions

In multicellular organisms, the elucidation of thermotolerance physiological mechanisms at cellular level can be as hard as impractical to accomplish. The utilization of *in vitro* cell systems has been a strategy for revealing cellular-associated responses that otherwise would be hidden at whole-plant level. Besides, cells in culture are relatively assessable and a large number of data per treatment could be easily achieved. However, for controlling the uniformity of environmental and physiological parameters, plant cell *in vitro* studies require the establishment of a fine stable suspension cell culture with a large proportion of small cell aggregates and single cells. A cell suspension culture was established from *P. euphratica* callus tissue. This suspension culture was subcultured every 12 days in fresh MS medium. The suspended cells grew vigorously with the passage of subculture, maintaining small clusters made up of a few cells. For evaluating cell growth, suspended cells aliquots (3 ml) were harvested every 2 days over a total period of 16 days (Figure 2.3). After a short adaptation period during the first two days of subculture, cell suspensions started an exponential growth phase that lasted for 8 more days, until day 10. Cell dry weight continued to increase until day 12, reaching eight-fold of the inoculum cell weight.

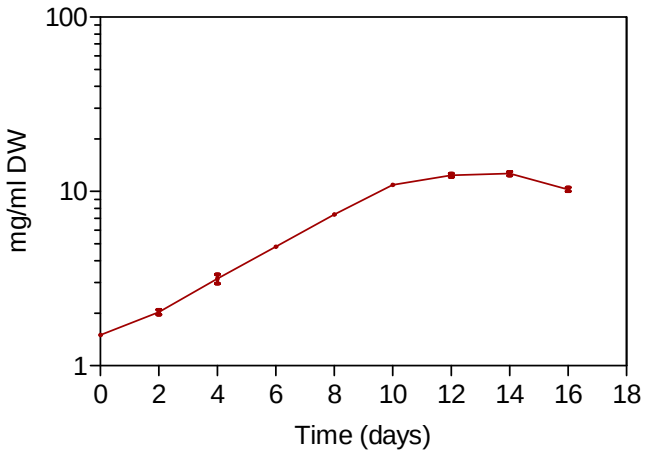

Figure 2.3. Growth curve of *P. euphratica* cell suspension culture. Subculture from a well-established suspension culture was evaluated for dry weight increase. Aliquots were harvested every 2 days for dry weight determination over a total period of 16 days. The results presented are mean values of three independent experiments ± SE.

At this point, cell suspensions appeared healthy when observed under microscope. After 12 days, the growth rate levels off and cell degeneration and necrosis become apparent (data not shown). These results indicate that cell suspensions should be cultured up to 12-day intervals for optimal growth. During exponential phase, the specific growth rate (μ) was 0.21 d^{-1}. For subsequent thermotolerance studies, where uniformity in the physiological state of the cells is required, cells would be used in the middle of exponential phase (day 5-6).

Heat stress induces cell death in *P. euphratica* suspended cells

In order to determine the temperature which results in cell death, the response of *P. euphratica* suspended cells to temperature stress was evaluated by subjecting suspension cultures to a range of temperature treatments (5-75°C). Analysis of cell viability was performed before the imposition of the 20 min temperature stress (0 h) and after recovery at 25°C for 10 min (0.5 h). During this period, cell viability was essentially unaffected for temperatures ranging from 5 to 45°C (Figure 2.4). Incubation at 50°C resulted in slightly decreased cell viability (89%), declining thereafter with increasing the temperature of the heat shock and reaching values near 25% for the highest temperature tested (75°C). When cell viability was evaluated 20 h after stress imposition, different values of cell viability were obtained from those obtained after 10 min of recovery (0.5 h) (Figure 2.4). A reduction in cell viability was observed in heat treatments at lower temperature values (40°C). Also, at higher temperatures cell viability upon 20 h of recovery is more reduced than after 0.5 h of recovery. Altogether, these results suggest that heat treatment not only have an immediate effect on cell viability, but also induces the cell death afterwards.

Cell viability was also analysed upon stress treatment and during recovery at 25°C, as a function of time over a total period up to 80 h (Figure 2.5). Temperature treatments between 5-35°C had no effect on cell viability (Figure 2.5A). In opposition, heat treatments at higher temperatures than 65°C promptly reduced the cell viability to ~20% immediately after the imposition of heat shock treatment. It is noteworthy to refer that a null viability value was never obtained, which is probably due to the inaccurate trypan blue viability assay due to the natural aggregation of plant cells.

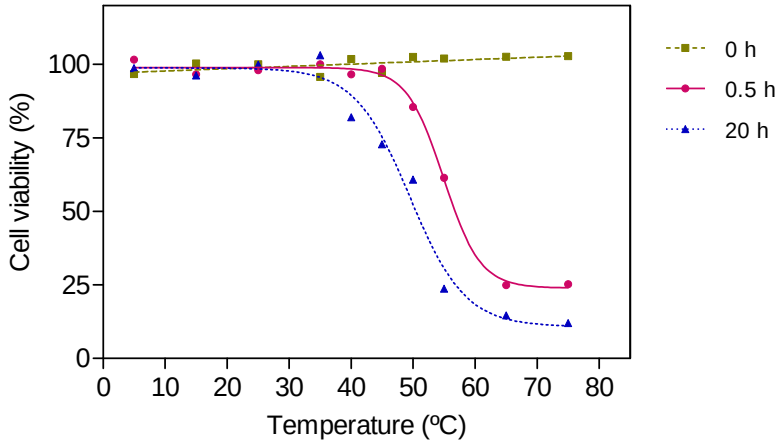

Figure 2.4. *P. euphratica* cell viability after different temperature stress treatments (5-75°C) of 20 min. Cell viability was determined by trypan blue exclusion method immediately before heat treatment (0 h), after recovery by 25°C incubation for 10 min (0.5 h) and during recovery (20 h) at growth conditions (25°C, with 125 rpm continuous shaking, in the dark). The results presented are mean values of twenty independent counts.

When temperatures of 45°C or 55°C were used, an induction of cell death was observed, as revealed by the long-time decrease on cell viability. In order to further analyse cell death induction, the same procedure was performed for temperatures between 40°C and 55°C (Figure 2.5B). Cell viability was significantly affected after heating at 55°C. Although cell viability still attained values of 72% after heat shock (0.5 h), it rapidly falls down to negligible levels after 10 h at recovery temperature (25°C). A more moderate cell death induction was observed after treatment with 50°C. Cell viability after heat shock (0.5 h) remained high (82%), but progressively declined to insignificant values after 75 h at 25°C. The specific death rates were determined for those heat-treated suspension cultures displaying cell death (40-75°C) (Table 2.1). The ability of cell growth recuperation was only observed after cell treatment at 40°C and 45°C. Immediately after heat shock (0.5 h), cells were not strongly affected, displaying still 91% and 87% of cell viability, respectively. However, an induction of cell death was evident till 45 h at recovery temperature, attaining cell viability values of ~75% and ~60%, for 40°C and 45°C treatments, respectively. After 45 h and until the end of the experiment, both cell suspensions were able to recover their growth ability, reaching the initial cell viability levels.

The results of cell suspension recovering ability were also evident when determining culture growth parameters after treatment at different temperatures (5-

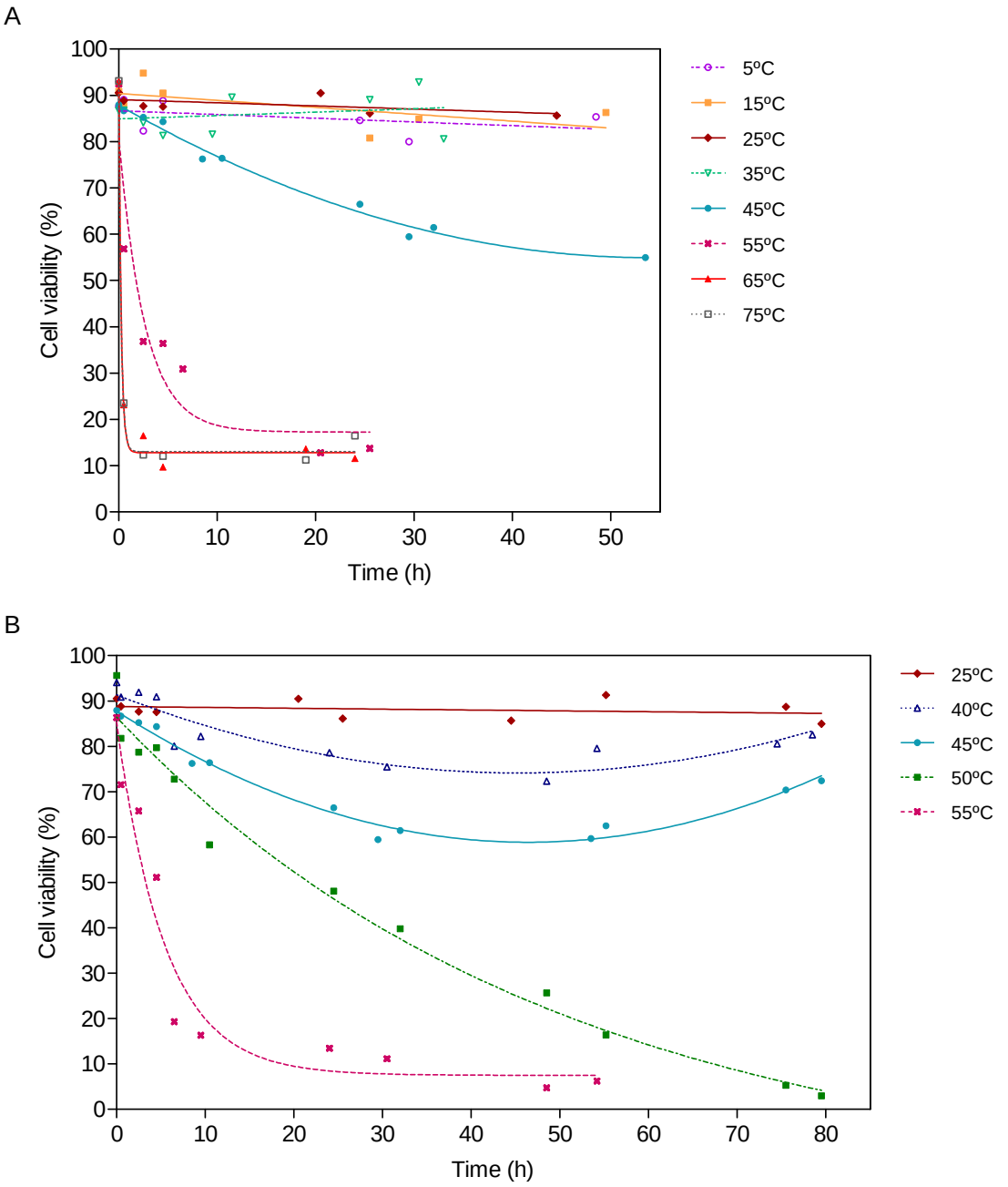

Figure 2.5. *P. euphratica* cell viability after incubation of cell suspensions for 20 min at different temperatures (5-75°C) and recovery at 25°C. Cell viability was determined by trypan blue exclusion method over a total period up to 54 h for heat treatments at 5-75°C (A) or over a total period up to 80 h for heat treatments at 25-55°C (B). The results presented are mean values of twenty independent counts.

65°C) (Figure 2.6). By following the time course of biomass content, it was observed that treatments with temperatures below 45°C did not affect the exponential growth phase of suspension cultures. The recovery capacity of cell suspensions suffering a 45°C treatment was again evident. After a declining period, corresponding to the first

Table 2.1 Specific death rates of *P. euphratica* cell suspensions after incubation for 20 min at different temperatures (25-75°C) and during recovery at 25°C.

Temperature	Specific death rate (h^{-1})
25°C	0
40°C	0.007
45°C	0.012
50°C	0.026
55°C	0.179
65°C	0.543
75°C	0.669

45 h after heat treatment as was previously shown (Figure 2.5), the biomass content showed a progressive increase and reached similar values than those obtained by control suspensions (25°C) but with a delay. In contrast, in suspension cultures incubated at 55 and 65°C, cell growth was completely impaired after treatment. According to these results, *P. euphratica* suspended cells also presented macroscopic differences after 24 h of heat treatment (Figure 2.7). Following temperature stresses higher than 45°C, cell suspensions became progressively darker and accumulated some debris, indicating cell lyses and death.

Considering the displayed results, we can conclude that *P. euphratica* suspended cells exhibited tolerance up to 45°C. Incubation at low temperatures (5 and

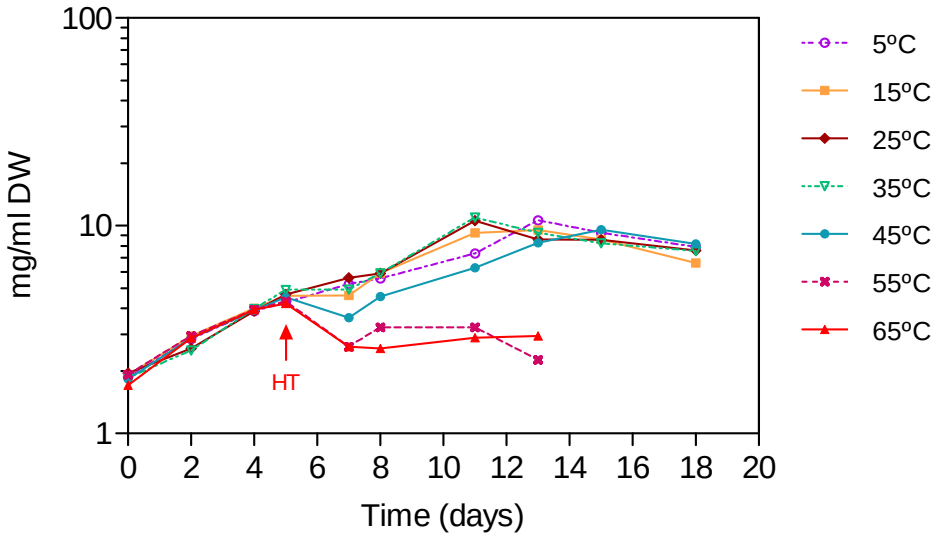

Figure 2.6. Dry weight of *P. euphratica* cell suspension cultures after incubation of cell suspensions for 20 min at different temperatures (5-65°C) and during recovery at 25°C. Aliquots were harvested for dry weight determination over a total period of 18 days. Heat treatment (HT) was performed in the middle of exponential growth phase (day 5).

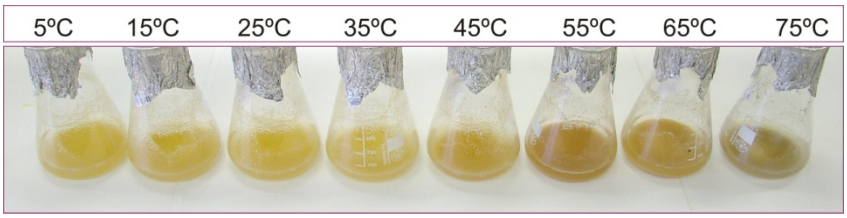

Figure 2.7. Macroscopic aspect of *P. euphratica* suspended cell cultures after stress treatment (20 min) at different temperatures (5-75°C), followed by 24 h at recovery temperature (25°C).

15°C) did not seem to affect cell viability, growth or appearance of cultured cells. These results suggest tolerance of cell suspensions to these chilling temperatures, at least for 20 min treatments. Previous studies in poplar species, performed with 3-month-old *in vitro* plants, have already demonstrated tolerance to a more extended chilling stress (4°C for 14 days) (Renaut *et al.* 2004; Renaut *et al.* 2005). Although growth rate was negatively affected, chilling tolerance was confirmed by the unaffected survival of the plants. Generally, plants from tropical and subtropical regions are chilling-sensitive, whereas those from temperate origins are chilling-resistant (Fowler *et al.* 2005). A few examples of agriculturally important sensitive cultivars are maize, rice, soybean, cucumber, tomato and tobacco, among others. At temperatures in the range of 5 to 15°C, chilling-sensitive plants suffer a variety of damages, such as the inhibition of germination and growth, tissue necrosis as well as the death of the whole plant. In maize, suspension-cultured cells started to suffer a decline in survival two days after initiation of chilling stress (4°C), resulting in less than 10% survival after 14 days of exposure (Xin and Li 1992). Tomato seedlings and suspension cultures equally displayed growth and viability reduction when exposed to chilling temperatures below 10°C for six days (Breidenbach and Waring 1977). In broad bean species, not documented as temperature tolerant, both chilling (5°C) and heat (42°C) stress induced a reduction in growth rate and membrane stability when applied to 21-day-old seedlings for 24 h (Hamada 2001). On the other hand, resistant plants as wheat, barley, spinach, pea, cabbage and *Arabidopsis* are not injured even by a low temperature of around 0°C. A retardation in the metabolism and an increase of membrane viscosity, as well as the accumulation of soluble carbohydrates for a putative cryoprotective process in leaves, have been suggested to be intrinsically linked to low temperature tolerance (Renaut *et al.* 2004; Renaut *et al.* 2005; Beck *et al.* 2007; Penfield 2008). In the present study, retardation in cell culture growth resulting

from the slowdown in metabolism did not occur, probably due to the short extent of temperature stress imposition (20 min).

When considering high temperatures, incubation of *P. euphratica* suspended cells at 35°C did not affect their cell viability or cell growth, presenting a similar behaviour as control cells (25°C). Fitness alterations in suspended cultures only appeared for temperatures higher than 40°C. A heat-induced decline in cell viability was observable at 40°C, but it was reversed during the recovery period. Although extended decrease in viability was found in 45°C treated-cells, accompanied by altered coloration of cell suspension, cell growth was still allowed to recover. In contrast, suspended cells treated at 50 or 55°C were not able to recover growth and a complete decline in cell viability was registered. In more extreme heat treatments (above 55°C), growth cessation and accelerated cell death occurred, which were probably due to cell membrane damage and necrosis. This result is in agreement with previous reports on *P. euphratica* leaf discs heat treated (50 and 55°C), for 30 min, that have shown membrane damage as evaluated by electrolyte leakage (Ferreira *et al.* 2006). It was also suggested that *P. euphratica* could tolerate temperature of 42°C for 54 h without survival effects. Using *P. euphratica* cell suspensions heat-shocked for 20 min, the temperature of 45°C corresponded to the highest sub-lethal temperature tolerated by cells.

Results from other plant cultured systems, identified as non tolerant species, exhibited quite similar responses. Although the results cannot be strictly comparable because the heat treatments were applied just for 10 min, the strongest induction of carrot and *Arabidopsis* cell death occurred for temperatures above 45°C, attaining maximum levels at 55°C (McCabe *et al.* 1997; McCabe and Leaver 2000). The same results were also registered using tobacco suspended cells heat treated for 20 min (Burbridge *et al.* 2007). In another assay performed with tobacco cell suspensions treated for 10 min, a time-dependent effect on cell viability was found after incubation at 55°C (Vacca *et al.* 2004). This response was very similar to the one observed in *P. euphratica* cultures subjected to 50°C for 20 min. Studies concerning the alterations of biological substances that are deeply related to temperature stress, such as HSPs, compatible solutes or membrane lipids, are essential for the elucidation of *P. euphratica* innate tolerance.

Programmed cell death as a process involved in heat response

This work suggested that heat treatment not only have an immediate effect on cell viability, but also induces the cell death. In *P. euphratica* suspended cells we identified three different types of heat response, based on cell viability analysis: a) at 40-45°C, a slightly decreased viability was recovered over time; b) at 50-55°C, a cell death process was induced progressively until negligible viability levels were reached; and c) above 55°C, an immediate decline in cell viability occurred upon heat treatment. Therefore, in addition to an uncontrolled necrosis, it is possible that a PCD process can be involved in inducing cell death, either in a) or b) responses. For this reason, *P. euphratica* cells heat-stressed at 45°C and 50°C were evaluated for typical morphological PCD-associated alterations. These temperatures seemed to be the most promising, since they resulted in a different response and degree of cell death during recovery. One of the most characteristic changes of plant PCD – the condensation of protoplasm away from the cell wall (Reape *et al.* 2008) - was detectable under visible light after trypan blue staining (Figure 2.8). A small number of heat (45°C or 50°C) stressed cells exhibited cytoplasm shrinkage after 24 h of stress treatment, whereas the untreated cells display a healthy living morphology. In the same stressed samples, we also observed some cells that did not display protoplast condensation but were trypan blue positive, suggesting the simultaneous occurrence of a necrotic death (data not shown). As already reported, heat treatment could activate both PCD or uncontrolled necrosis in carrot, *Arabidopsis*, soybean or tobacco cell cultures (McCabe *et al.* 1997; McCabe and Leaver 2000; Vacca *et al.* 2004; Zuppini *et al.* 2006; Burbridge *et al.* 2007). While a necrotic cell death is seen as a chaotic and involuntary response resulting from a permanent collapse of cell membranes, programmed cell death is a highly organized and controlled event. Accordingly, when exposed to extreme temperatures for a relatively short time, programmed cell death can be triggered, and typical morphological features such as time-dependent cleavage of genomic DNA, nuclear condensation and protoplast retraction away from the cell wall are observed (Reape and McCabe 2008). Suspended cells from the referred plant species were incubated at different levels of heat stress and cell death occurred with a very distinctive protoplast retraction in temperatures up to 55°C. The occurrence of this

Figure 2.8. Cell death morphology of *P. euphratica* suspended cells subjected to temperature treatments. The morphological changes were observed after trypan blue staining under phase contrast in untreated cells (A) and in 45°C (B) and 50°C (C) treated cells, 24 h after stress imposition. Alive cells were able to exclude the trypan blue stain and remained uncoloured. In dead cells that undergo PCD, trypan blue permeated the damaged membrane and protoplast retracted leaving a visible gap between membrane and cell wall. Necrotic cells appeared stained but showed no evidence of protoplast condensation. (400x).

feature reduced progressively for higher temperatures and was absent in cells that died with treatments above 75°C, which confirmed that protoplast retraction observed at temperatures up to 55°C was due to a PCD process. McCabe *et al.* (1997) have developed a temperature inducible cell death assay where they distinguished living cells, those undergoing PCD and necrotic cells through analysis of cell morphology. Morphological alterations can be observable 1-3 h after treatment, but are easier to distinguish when protoplast reaches its maximal condensation (after 12-24 h). This assay has been used to score subtle changes in cell death rates of *Arabidopsis* and carrot (McCabe *et al.* 1997; McCabe and Leaver 2000; García-Heredia *et al.* 2008) and tobacco (Vacca *et al.* 2004; Burbridge *et al.* 2007) cultures. Although clearly observable, no differences in the amount of undergone PCD or necrotic cells were detected in this work and the retraction feature was not used to quantitatively discriminate between both types of cell death in *P. euphratica*. Although almost all previous works were performed with a shorter treatment (10 min), all plant species depicted a near complete cell death 24 h after 55°C treatment. This death was mainly the result from a PCD process, detected by cytoplasm shrinkage, chromatin condensation and DNA laddering. Some extent of necrotic death was also found in these conditions in *Arabidopsis* and tobacco (McCabe and Leaver 2000; Vacca *et al.* 2004). According to the results published by Burbridge *et al.* (2007), tobacco cell cultures subjected to 50°C for 20 min (in the same conditions as applied in *P. euphratica* heat treatment) displayed ~70% of cell death 24 h after treatment. Analysis of cellular and nuclear morphology allowed identification of PCD-associated

features in all tobacco cells, suggesting that cell death was completely due to PCD induction. In *P. euphratica* cell suspensions treated at 45°C and 50°C (20 min), both PCD and necrotic cell levels were found, suggesting the occurrence of both cell death processes.

In order to quantitatively evaluate the occurrence of PCD, we assayed the level of DNA degradation in the same heat-stressed cells. In the last stages of PCD, cleavage of DNA at specific chromosomal sites by endonucleases results in exposed 3'-hydroxyl ends, which can be fluorescently labelled with the TUNEL (Reape and McCabe 2008). However, a positive labelling result should be taken carefully, since necrotic cells can also display random DNA cleavage. When TUNEL staining was employed to ascertain whether a short time heat stress can induce PCD in cultured cells of *P. euphratica*, low unspecific TUNEL staining was observed in treated cells (50°C, 20 min) allowed to recover at 25°C for 8 h (Figure 2.9). This result suggests that the most part of the dead cells had probably died necrotically, even though some extent of morphological changes was registered lately in recovery (24 h). A similar result was obtained for 45°C treated cells (results not shown), being only observed a diffuse labelling rather than the characteristic nuclear staining similar to what was observed in the positive control (Figure 2.9C).

DNA degradation during heat-induced PCD depends on the activation of endonucleases, which occurs in a later stage of the process. DNA cleavage detected by the observation of a DNA laddering in an agarose gel has been reported in several abiotic stress-induced PCD, as reviewed by Reape and McCabe (2008). Although in carrot cell cultures, detection of DNA fragmentation was possible 5 h after heat

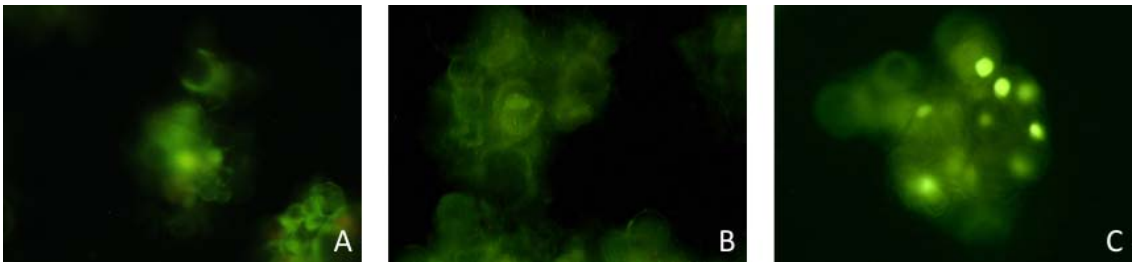

Figure 2.9. Programmed Cell Death evaluation by TUNEL assay in heat stressed *P. euphratica* suspended cells. Cell suspensions were labelled before stress (A) and 8 h after 50°C treatment (20 min) and recovery at 25°C (B) and observed using fluorescence microscopy (UV light, 495/529 nm). A positive control was carried out (C). The TUNEL assay was performed using the In Situ *Cell Death Detection Kit – Fluorescein* (Roche Applied Science). Pictures represent typical examples (250x).

treatment (55°C, 10 min), using either TUNEL or DNA laddering assays, in the majority of the referred species (cucumber, *Arabidopsis* and tobacco) DNA fragmentation was only visualized 12-72 h after the PCD-inducing treatment. A specific TUNEL staining was also only observable 12 h after acetylsalicylic acid treatment of *Arabidopsis* cultures (García-Heredia *et al.* 2008). Additionally, for *Arabidopsis* and tobacco, the evaluation of DNA degradation was performed after a period that resulted in nearly 100% of cell death which increases the magnitude of response. In heat-stressed (55°C) *Arabidopsis* (McCabe and Leaver 2000) and tobacco cells (Burbridge *et al.* 2007), for 10 and 20 min, respectively, DNA laddering was only detectable 24 h after heat treatment, when all cells were dead. Also in heat-stressed (55°C, 10 min) tobacco cells, DNA laddering was detectable between 48 and 72 h, at which times 75% to 98% cells were already dead (Vacca *et al.* 2004). In *P. euphratica* suspended cells, 8 h after recovery from 45 and 50°C treatments, cell viability was respectively ~22% and ~30% and TUNEL labelling was similar to untreated cells. This result suggests that PCD detection by TUNEL assay should had been performed in a situation that causes a higher percentage of cell death (upon 55°C treatment) or in a latter phase of recovery (at least 12 h after treatment).

ROS production in heat-stressed suspended cells

One of the most notorious immediate effects on temperature-stressed cells is the dramatic levels of toxic compounds accumulation, in which ROS can be included (reviewed by Suzuki and Mittler 2006). Although the underlying role of ROS as signaling molecules during abiotic stress has been covered for a long time by its inherent toxic effect, it is now widely accepted the dual function of ROS as signaling and destructive effectors (Van Breusegem and Dat 2006). In recent studies, the involvement of ROS in signal transduction for the activation of stress-associated pathways and induction of protective mechanisms was established (reviewed by Suzuki and Mittler 2006). Plant heat tolerance results from stress perception and defense by ROS. The ROS gene network is then interlinked with other dissimilar networks that control thermotolerance. Accordingly, the degree of thermotolerance depicted by a given plant was suggested to be extremely connected with ROS scavenging.

Considering the crucial role of ROS, we investigated their production in heat-shocked *P. euphratica* suspended cells by following the fluorescence of the reduced fluorescein compound. Cells were labelled before and just after heat stress treatment (45 or 50°C) and during recovery at 25°C (2, 6, 12 and 29 h). The detection of the bright green fluorescence resulting from the presence of non-specific ROS was negligible in all observed samples, as displayed in Figure 2.10 for heat-shocked cells at 50°C (data from 45°C stress not shown). No more than a residual 2% of stained cells were observed in all conditions. The results contrast with previous reports that have detected an enhanced production of ROS with several stresses, such as high light, wounding, drought, UV or extreme temperatures (Neill *et al.* 2002). Particularly, in cells under heat stress a burst of superoxide and/or hydrogen peroxide was reported to occur (Larkindale and Knight 2002; Vacca *et al.* 2004; Kotak *et al.* 2007). ROS overproduction may affect pathways that are switched on in response to heat stress, which could be responsible for enhancing HSP production and lead to thermotolerance. Although incubation (20 min) at 45 or 50°C of *P. euphratica* cells did not dramatically produced

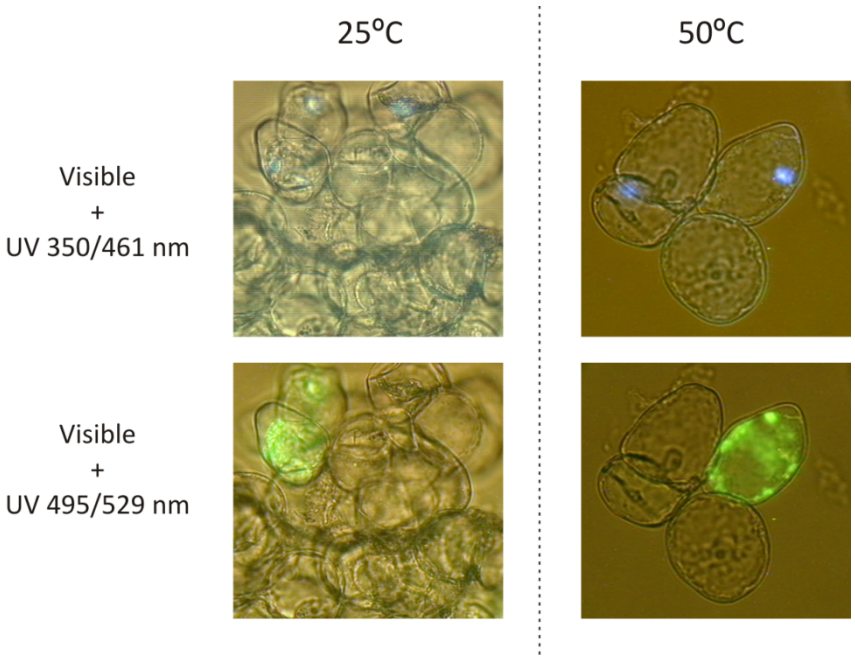

Figure 2.10. ROS production in heat-stressed *P. euphratica* suspended cells. Cell suspensions were labelled before stress and 6 h after 50°C treatment (20 min) and recovery at 25°C and observed using fluorescence microscopy for the detection of nuclei (visible light + UV light, 350/461 nm) and ROS production (visible light + UV light, 495/529 nm). Detection of nuclei was performed by using the Hoechst 33342 (Molecular Probes) nuclear staining and ROS production was detected by *Image-iT LIVE Green ROS Detection Kit* (Molecular Probes). As control, untreated cells (25°C) were also assayed. Pictures represent typical examples (400x).

increased levels of ROS, a similar experiment performed in tobacco cell suspensions showed that an incubation at 55°C for 10 min had enhanced considerably the production of ROS immediately after heat treatment (Vacca *et al.* 2004). This result suggested that the amount of accumulated cellular ROS is critical and the increase in ROS is a prerequisite for heat-induced PCD. However, it should be noted that a certain threshold level of ROS is essential to induce PCD, by activation of the signal transduction pathway, but higher doses, resulted rapidly in cell death via necrosis (Van Breusegem and Dat 2006). In heat-stressed (45 and 50°C) *P. euphratica* cells, few ROS producing cells were detected, in equal amounts as in untreated cells. This result is in agreement with those achieved for PCD assay, since a reduced level of ROS would not trigger PCD. Accordingly, the results suggest that heat induced cell death may had occurred mainly via necrosis in 45 and 50°C stressed *P. euphratica* cells. However, whether temperature induces or not a PCD process and overproduction of ROS in *P. euphratica* suspended cells is far from being elucidated and further investigation is needed.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this work, the high natural tolerance of the model tree *P. euphratica* (up to 54°C) was not clearly evidenced by the viability assays performed in suspended cells. Similar results have been reported in plant species which normally cannot manage such high environmental temperatures as those tolerated by *P. euphratica in vivo* plants. This can occur due to the higher sensibility of suspended cells in sensing temperature alterations, when compared to whole plants growing in the field. In addition, a suspended cell alone or within few cell aggregates would not have the same ability to cope with a heat-stress situation as a cell integrated into a system of multiple interacting cells. It is only when integrated into a tissue that cells would probably achieve the capacity to serve as fundamental units of tissue homeostasis and repair during stressful situations. Therefore, it would be expected that the thermotolerance mechanisms activated by elevated temperatures in nature maybe induced by lower temperature levels in cell suspensions. Cell viability recuperation observed for heat treatments at 40 and 45°C (20 min) followed by long recovery periods (> 45 h) suggests thermotolerance capacity following heat treatments at these temperatures. This was confirmed by the increasing levels of biomass along the recovery period for cells treated with temperatures up to 45°C. Heat stress has been reported as having a high detrimental effect on the growth and metabolism of plants in their natural habitats. For *P. euphratica in vitro* plants, although no effects in survival were verified after prolonged exposure to 42°C, growth arrestment was evident probably due to the reduction of photosynthesis ability (Ferreira *et al.* 2006). In suspended cells, growth or metabolism reduction for temperatures up to 45°C were not observed.

In this work, it was evident that *P. euphratica* cell suspension heat treatments above 40°C for 20 min, not only have an immediate effect on cell viability, but also induces the cell death afterwards. This observation has been already suggested by an increase of cell membrane ion permeability in *P. euphratica* leaf discs following treatment at 50 or 55°C for 30 min (Ferreira *et al.* 2006). Apart from all the inherent questions that still have to be clarified, the results from PCD activation and ROS production brings the hypothesis that these mechanisms were not implicated in the

referred cell death induction. As mentioned, further clarifying approaches are necessary, such as considering the occurrence of these events after treatment at 55°C.

Other morphological or physiological features, not considered as part of the temperature tolerance machinery, might also be responsible for the natural tolerance of *P. euphratica*. It has already been demonstrated that the strong capability to tolerate drought stress presented by this species is mostly due to the development of effective roots to access deep water tables (Gries *et al.* 2003). Also, the adaptation to high salinity environments appears to be related to the ability to adapt to higher osmotic stress by keeping cell integrity and effectively controlling ionic toxicity (Gu *et al.* 2004*b*). It should also be noted that *in vivo*, plants encounters a combination of several abiotic stresses rather than one individually. In drought stricken areas, like the natural habitat of *P. euphratica*, a combination of drought and other stresses, such as heat or salinity, are encountered and an integrated and unique response is employed by plants, as the product of several interconnected responses (Knight and Knight 2001; Mittler 2006). Therefore, we cannot exclude the possibility that temperature tolerance presented by *P. euphratica* in the field, demonstrated by the absence of related injuries, can be due to the capacity to achieve morphological or physiological modifications and/or overcome related stresses rather than specific thermotolerance mechanisms.

In the future, different approaches should be applied taking advantage on recent developed genomic and molecular tools for *Populus* (genome sequence, EST collections, DNA microarrays, transformation protocols, etc) and also on the phylogenetic proximity with *Arabidopsis*. The combination of genomic, proteomic and physiological approaches offers new possibilities tending to elucidate the function of temperature stress machinery.

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Chapter 3

Identification of stress determinants in *Arabidopsis*: a reverse genetics strategy

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*Both authors contributed equally to this work.

CONTENTS

Identification of stress determinants in *Arabidopsis*: a reverse genetics strategy

ABSTRACT

Since completion in 2000 of the *Arabidopsis thaliana* genome sequencing, plant researchers all over the world have faced the complex challenge of assigning a function to thousands genes. Functional determination of a large fraction of the deduced genes was achieved automatically by sequence similarity, but only a minor part was experimentally validated. To facilitate gene function discovery, collaborations between several functional genomics laboratories led to a massive increase on the availability of *A. thaliana* genomics resources. As more and more tools and resources are being developed and improved for functional *Arabidopsis* studies, the systematic assignment of gene function is becoming increasingly accessible. In the present work, we propose a straightforward strategy supported by reverse genetics principles that allows for the identification of novel stress-related determinant genes. Initially sustained by gene expression data acquired in publicly available microarray experiments, this approach makes use of the numerous web-based resources that are currently accessible. An overview of the several promising tools and databases for functional research is presented. The feasible application of the outlined strategy is illustrated by the identification of novel determinant genes in a specific heat-associated practical case.

KEYWORDS *Arabidopsis thaliana* · Bioinformatics · Functional genomics · Reverse genetics · Thermotolerance

ABBREVIATIONS ABI3: **ab**scisic acid **i**nsensitive **3** · ABRC: ***A**rabidopsis **B**iological **R**esource **C**enter* · AGRIKOLA: ***A**rabidopsis **G**enomic **R**NA**i** **K**nock-**o**ut **L**ine **A**nalysis* · AGRIS: ***A**rabidopsis **G**ene **R**egulatory **I**nformation **S**ervice* · AP2: **ap**etala **2** · ARAMEMNON: *Plant Membrane Protein Database* · AREX: ***Ar**abidopsis Gene **Ex**pression Database* · AtcisDB: ***A**rabidopsis **t**haliana **cis**-regulatory **Data**base* · AthCoR: ***A**. **th**aliana **Co**-**R**esponse Database* · ATIDB: ***A**rabidopsis **t**haliana **I**ntegrated **Data**base* · ATTED: ***A**rabidopsis **t**haliana **T**rans-factor and cis-**E**lement prediction **D**atabase* · *AtTERT*: ***A**. **t**haliana* **TERT** gene · AtTFDB: ***A**rabidopsis **t**haliana **T**ranscription **F**actor **Data**base* · BAR: ***B**io-**A**rray **R**esource* · RIKEN BRC: ***RIKEN B**io**R**esource **C**enter* · CATMA: ***C**omplete **A**rabidopsis **T**ranscriptome **M**icro**A**rray* · CSB.DB: ***C**omprehensive **S**ystems-**B**iology **Data**base* · DAG1: **D**of **a**ffecting **g**ermination **1** · DNA: **d**eoxyribo**n**ucleic **a**cid · eFP: **e**lectronic **F**luorescent **P**ictographic · EMS: **e**thane **m**ethyl **s**ulfonate · FST: **f**lanking **s**equence **t**ag · GO: ***G**ene **O**ntology Project* · GST: **g**ene-specific **s**equence **t**ag · HRR: **h**eat **R**NA-**r**ecognition motif containing protein · HSE: **h**eat **s**hock **e**lement · HSFA2: **h**eat **s**hock transcription **f**actor **A2** · HZF: **h**eat **z**inc **f**inger family protein · INRA: ***I**nstitut **N**ational de la **R**echerche **A**gronomique* · MAtDB: ***M**IPS **A**rabidopsis **t**haliana **Data**base* · MIAME: ***M**inimum **I**nformation **A**bout a **M**icroarray **E**xperiment* · MIPS: ***M**unich **I**nformation Center for **P**rotein **S**equence* · NASC: ***N**ottingham **A**rabidopsis **S**tock **C**enter* · PCR: **p**olymerase **c**hain **r**eaction · RAV1: **r**elated to **A**BI3/**V**P1 **1** · RING: **r**eally **i**nteresting **n**ew **g**ene · RNA: **r**ibo**n**ucleic **a**cid · RNAi: **RNA i**nterference · SIGnAL: ***S**alk **I**nstitute **Gen**omic **A**nalysis **L**aboratory* · TAIR: ***T**he **A**rabidopsis **I**nformation **R**esource* · T-DNA: **t**ransferred **DNA** · TERT: **te**lomerase **r**everse **t**ranscriptase · TILLING: ***T**argeting **I**nduced **L**ocal **L**esions **In G**enomes* · VP1: **v**ivi**p**arous **1**

BACKGROUND ON FUNCTIONAL GENOMICS

In the wake of the international coordinated effort to sequence the first genome of a higher plant (Arabidopsis Genome Initiative 2000), plant research entered in a stimulating post-genomic era. The application of experimental approaches to elucidate gene function is now becoming an essential part of plant research. The heading focus on *Arabidopsis thaliana* research is presently to assign the biological function of thousands of predicted genes, making use of the data and materials afforded by structural genomics. Besides presenting a small genome, the model plant *A. thaliana* also displays powerful attributes for gene function assessment: a fast and condensed growth and simplicity of genetic manipulation, together with the efficient ability to be transformed with *Agrobacterium*. Also, a variety of new resources (databases, analysis tools, DNA, seed and mutant lines collections) is currently available (Table 3.1), which have resulted from a well organized scientific community (Feng and Mundy 2006).

After identifying a new gene sequence, homology search in sequence databases is the easiest way to attain further critical biological information. Such *in silico* analysis is limited by the lack of homology of a large number of sequences to any known genes, or insufficient sequence similarity to infer gene function (Sessions *et al.* 2002). The recent review from Alonso and Ecker (2006) provides an excellent overview on the basis of functional genomics for understanding the function of unknown genes. Within phenotype-centered approaches, forward and reverse genetics are two different pathways for achieving the same end. Forward techniques have been the earlier extensively employed strategy to determine gene function. Systematic screening for a detectable phenotype originated by inducing gene mutation is followed by cloning the gene responsible for it. Thus, this approach starts with the selection of a biological process of interest and is directed from phenotype identification to gene isolation. In contrast, a reverse genetics strategy goes from gene selection to detection of a visible phenotype. Once a gene of interest is identified, generation of a mutant line with altered gene expression is required to initiate screening of a potential phenotype. Strategies can include the generation of overexpression lines or RNAi-based gene

Table 3.1 Bioinformatics resources for *A. thaliana* functional genomics.

Resource/Database	Brief description
General resources	
TAIR [*http://www.arabidopsis.org*]	Provides access to data about genes, markers, maps, sequences, gene families and proteins, clones, DNA and seed stocks, polymorphisms, publications and information about *Arabidopsis* researchers
MatDB [*http://mips.gsf.de/proj/thal/db*]	Allows access to the sequence data of the *Arabidopsis* Genome Initiative; sequences have been assembled, analyzed, annotated, and stored at MIPS
Arabidopsis web services [*http://bioinfo.mpiz-koeln.mpg.de/araws*]	Pilot project created to improve the availability and integration of *Arabidopsis* data residing at many different locations, through a network of standardized web service providers
Plant material and stock centers	
NASC [*http://arabidopsis.info/*]	Collects and distributes seeds to Europe along with information resources in a coordinated activity with ABRC
ABRC (Distribution through TAIR)	Collects and distributes seeds, DNA clones and libraries to North America; the ordering is made through TAIR
FLAG [*http://dbsgap.versailles.inra.fr/publiclines*]	Collects and distributes a set of T-DNA insertion mutants, more than 500 ecotypes and several recombinant inbred line populations created in the Versailles resource center (INRA)
AGRIKOLA (Distribution through NASC)	Biological resources from the AGRIKOLA project (GSTs entry clones, pAGRIKOLA clones and RNAi lines) are distributed through NASC
GABI-Kat [*http://www.gabi-kat.de/*]	Distributes confirmed T-DNA insertion mutants characterized by FSTs in Columbia ecotype; these lines are in process of donation to NASC
RIKEN BRC [*http://www.brc.riken.jp/lab/epd/Eng/*]	Preserves and distributes T-DNA/transposon mutant seeds and cDNAs which were independently developed in RIKEN BRC
LEHLE SEEDS [*http://www.arabidopsis.com/*]	Distributes a collection of mutant seeds produced with EMS, fast neutrons or gamma-rays
Gene knockout mutants and knockdown lines	
ATIDB [*http://atidb.org/*]	Database which integrates general sequence and molecular *Arabidopsis* information along with T-DNA/transposon lines and additional data generated at the John Innes Center
SIGnAL T-DNA Express [*http://signal.salk.edu/cgi-bin/tdnaexpress*]	Platform for searching the insertion sites localization and sequences in a large collection of mutant lines, using a simple genome map-based graphical interface
SIGnAL T-DNA Primer Design [*http://signal.salk.edu/tdnaprimers.2.html*]	PCR primer designing tool for efficient screen of homozygous lines in insertion mutants; outcomes include insertion site localization, primers sequence and estimated product size
AGRIKOLA [*http://www.agrikola.org/index.php?o=/agrikola/main*]	Provides information about the project developed for the creation of a collection of silenced lines using CATMA GSTs, using a high-throughput recombinational cloning technique
CATMA [*http://www.catma.org/*]	Provides information about the GSTs probes, designed and produced by the CATMA consortium; provides information about the genes to which GST hybridize and the PCR primers used for their amplification

Table 3.1 (continuation)

Resource/Database	Brief description
Additional gene information	
ATTED-II [*http://www.atted.bio.titech.ac.jp/*]	Database for prediction of *cis*-elements and co-regulated genes of a particular gene and graphical visualization of gene expression; provides co-expression gene interactions intended for function inference
AthCoR@CSB.DB [*http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath.html*]	Resource of transcriptional co-response information based on filtration of the best co-regulations among transcript level variation
AGRIS [*http://arabidopsis.med.ohio-state.edu/*]	Assembles information on promoter sequences, transcription factors and their target genes; comprises two independent databases (AtcisDB and AtTFDB) functioning in *tandem*
ARAMEMNON [*http://aramemnon.botanik.uni-koeln.de/*]	Database for searching putative plant membrane proteins and several related informational features
AZTEC [*http://gfp.stanford.edu/*]	Project currently in progress, proposed to evaluate native expression patterns and subcellular localization of functionally undetermined gene products, using large-scale fluorescent tagging
Microarray data/gene expression analysis	
NASCArrays [*http://affymetrix.arabidopsis.info/*]	Provides access to results from *Arabidopsis* microarray experiments mainly run by the NASC Affymetrix Facility; the database also includes experimental data from other centers
ArrayExpress [*http://www.ebi.ac.uk/microarray-as/ae/*]	Repository for transcriptomics data, available for browsing, querying and retrieval of specific experiment properties
Genevestigator [*https://www.genevestigator.ethz.ch/*]	Expression database including a suite of user-friendly tools that convert data into easily interpretable results; allows the study of expression and regulation of genes in a broad variety of contexts
BAR [*http://www.bar.utoronto.ca/*]	Subset of tools designed for expression, genomic and molecular marker analysis; specifically suitable for functional genomics research
AREX [*http://www.arexdb.org/index.jsp*]	Stores spatial gene expression data derived from Affymetrix microarrays and gene-specific experiments (*in situ*, GFP-reporter, etc.); only root data is currently available

knockdown, but frequently rely on insertion mutant lines that interrupt target genes via biological vectors. The substantial increase in the number of gene-indexed T-DNA/transposon mutant collections, now covering most of the *Arabidopsis* genome, has turned reverse genetic approaches into an attractive alternative to forward genetic screens. Collections can be easily browsed and mutant seeds obtained from stock centers (Table 3.1). Nonetheless, despite forward and reverse strategies being viewed as independent approaches to study gene-function relationships, they have already been elegantly combined in a successful way (Dekker 2005).

For phenotypic effect analysis of perturbing gene activity, both reverse and forward approaches make use of highly redundant mutant populations that can be obtained by standard chemical/physical or biological mutagenesis (Alonso and Ecker 2006). While chemical mutagenesis is the method of choice in forward genetics, gene disruption by introducing a biological element (T-DNA or transposon) is the predominant technique in a reverse strategy. In this case, the random mutagenized populations are produced using biological agents that mediate DNA transfer into plants (*e.g.*, *Agrobacterium*-mediated transformation). Sequencing of a large bunch of insertion sites results in a gene-indexed catalogue of mutants. As previously stated, advantage can now be taken from the large available collections of knockout lines produced by several independent laboratories. As reviewed by Feng and Mundy (2006), collections were made available by arisen consortiums, differing mainly in the type of insertion element and the plant ecotype used. Several databases and tools are available in the web for searching mapped insertion sites of the majority of the insertion mutant lines (Table 3.1). After finding the mutant of interest, researchers can simply order the corresponding seeds from a public stock center. As the disruption of the gene sequence could drastically alter or even abolish gene function, a gene-phenotype connection could be deduced by evaluating the fitness of mutant plants. A number of alternative knockdown/knockout strategies are also currently available for reverse genetics, such as RNAi, TILLING or Deleteagene, the use of which has been growing in recent years (Alonso and Ecker 2006; Eamens *et al.* 2008; Gregory *et al.* 2008). The above mentioned functional genomics tools established for *Arabidopsis* research, either using a forward or reverse genetics strategy, are summarized in Figure 3.1.

While the access to a mutant line of interest may be an undemanding step in a reverse genetics approach, the search for a phenotype is not quite as simple. For most *Arabidopsis* knockout lines used for functional inference, a visible and directly informative phenotype was not detected. Additionally, from the successful cases that have been reported, only few have a detailed description of phenotypic analysis. One representative example of the efficiency of a knockout mutant phenotype in providing a direct clue to gene function was the work concerning *DAG1*, a gene that encodes a

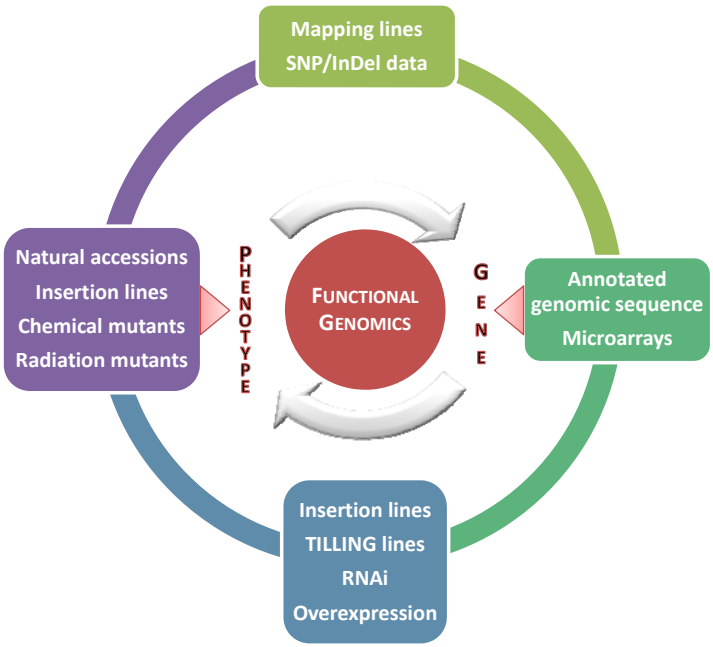


Figure 3.1

General outline of currently available *Arabidopsis* functional genomics tools (adapted from Dekker 2005).

Dof zinc finger transcription factor (Papi *et al.* 2000). The involvement of this gene in the control of seed dormancy was inferred by phenotypic analysis of the homozygous knockout mutant. Unlike wild-type, mutant plants displayed twisted siliques and produced seeds that did not develop dormancy and were capable of germinating in total darkness. Although the existence of successful cases, finding and studying informative phenotypes can be an unpredictable task, since slight morphological alterations as well as structural redundancy may mask gene function. Hence, evaluation of phenotypic alterations resulting from gene disruption is not adequate by itself to elucidate gene function (Bouché and Bouchez 2001).

Indirect information on biological function of a particular gene can be obtained from its spatial and/or temporal expression patterns. The recent advances in DNA microarray technology have generated a powerful tool for rapidly increasing the current knowledge of gene functional roles (Figure 3.2). Large sets of experimental data were developed for expression analysis in different *Arabidopsis* tissues, during developmental stages and in response to numerous treatments and experimental conditions. A number of *Arabidopsis* genome-wide expression datasets were then

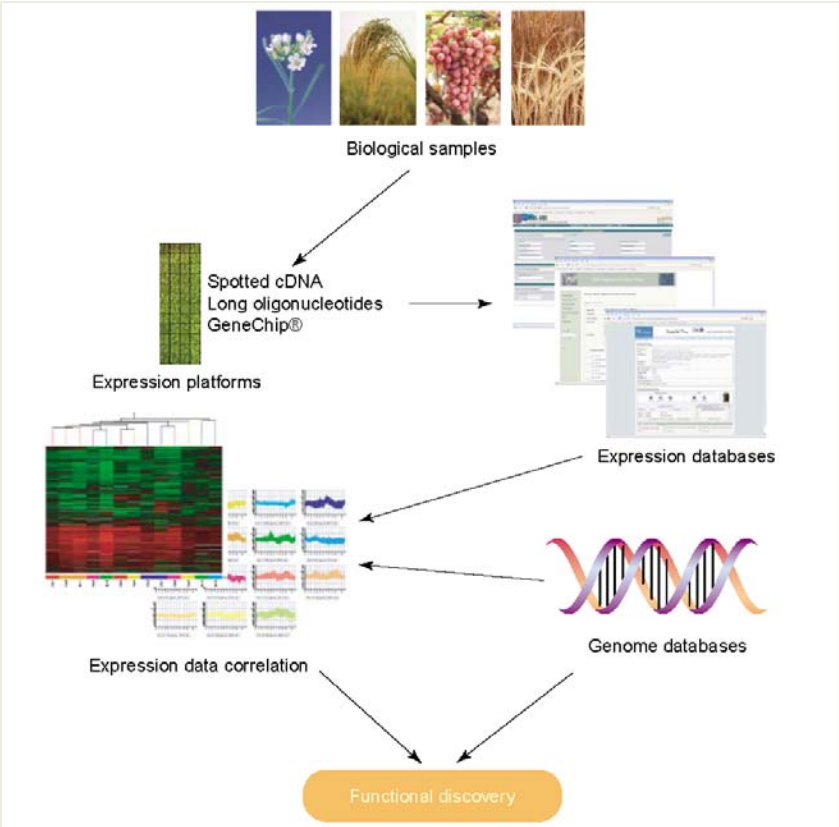


Figure 3.2

The use of expression data analysis in functional genomics. Transcription profile experiments provide expression data from several plant species by using different approaches (illustrated in the figure by the indexed array GeneChip system used by Affymetrix), which are integrated in publicly available expression databases. Prediction of putative gene functions can be achieved by analysis of expression profiles and integration with existing genome sequences and annotation data (Rensink and Buell 2005).

produced and made publicly available by a microarray network (Table 3.1). Additionally, abundant global information could be assembled by using a number of freely accessible informative resources (Table 3.1). Sequence annotation, transcript expression patterns, co-expressed genes and other existing supplementary information could be of extreme assistance in the functional characterization of the gene of interest. The use of such web-based resources frequently switches the stressing and time-consuming search for a phenotype into a hypothesis supported research. The effectiveness of this type of information in helping to access gene function was demonstrated in some examples highlighted by Bouché and Bouchez (2001). For example, the function of a telomerase gene (*AtTERT*) was first suggested by the presence of motifs conserved in all other characterized TERT proteins (Fitzgerald *et*

al. 1999). The disruption of the *AtTERT* gene, which results in shorter telomeres in knockout plants, was not sufficient for functional inference since no apparent morphological alteration was present for ten generations.

Although some pending issues still remain, the summarized amount of data and resources generated for *A. thaliana*, together with its relative straightforwardness, turned reverse genetics into the currently predominant used approach for determining gene-function relationships. The tremendous amount of data generated by cooperation projects and additional global independent research led to the enlargement of currently available web-based portals directed to *Arabidopsis* research. A short overview on the various bioinformatic resources with valuable application facing the reverse genetics approach is subsequently performed. Not pretending to be a comprehensive list of *Arabidopsis*-related web portals, a summarized table is presented that focuses on these resources (Table 3.1).

General *Arabidopsis* resources

By their informative and correlative character, general resources are essential valuable tools. One of the most generally explored on-line databases is *The Arabidopsis Information Resource* (TAIR). Genetic, physiological and biochemical information assembled for this plant model is combined with several tools, analysis and visualization potentialities (Huala *et al.* 2001; Garcia-Hernandez *et al.* 2002; Rhee *et al.* 2003; Swarbreck *et al.* 2008). This comprehensive and updated biological knowledge resource integrates complete information on gene families, gene product information, metabolism and expression data, DNA and seed stocks, genetic and physical markers along with genome maps, sequence polymorphisms (including alleles, germplasms and phenotypes), *Gene Ontology* (GO) annotations, research papers and members of the *Arabidopsis* research community. Additionally, external links for useful connections are provided. Through an intuitive interface, TAIR repository has become an attractive and mandatory tool for *Arabidopsis* functional research.

Another general resource generated in the post-genome era, the *MIPS Arabidopsis thaliana Database* (MAtDB), was originally intended as a repository for the sequence data collected by the genome sequencing initiative (Schoof *et al.* 2002). Further improvements were performed to transform this resource into an integrated

knowledge database, gathering genome sequence, several datasets and advanced tools to search and analyze the information. Besides its importance as an *Arabidopsis* tool, MAtDB also assists in the transmission of knowledge to other species by annotation alignments, thus enhancing the significance of *Arabidopsis* data.

Although bioinformatic resources are powerful ways of getting useful information on *Arabidopsis* gene function, they still present several limitations (reviewed by Rhee *et al.* 2006). Datasets generated by separate laboratories are often not easy to integrate due to the diversity of data formats and protocols, growing conditions and inappropriate sample documentation, which slows down the progress on biological research. As an attempt to incorporate functional genomics efforts, a pilot project has been developed to overcome the difficulties of fragmented, disseminated and multiplied data. Assembling several European and American partners, the project *Webservices for Arabidopsis data integration* pretends to establish a connection between several standardized web resource suppliers, leading to a more accurate service.

Plant material and stock centers

Stock centers are key resources for *Arabidopsis* research, allowing for storage and distribution of plant material generated by different research groups. The main public collection of *Arabidopsis* seeds is available for order in NASC (Scholl *et al.* 2000). NASC stocks include characterized ecotype lines, mapping populations and a large number of transgenic and knockout mutant lines donated by several laboratories all over the world. In this group are also included the AGRIKOLA resources. Another major stock center, the *Arabidopsis Biological Resource Center* (ABRC), assembles and provides seeds and DNA collections which can be browsed in TAIR webpage. In a coordinated activity, these stock centers separately distribute plant resources to Europe (NASC) and to North America (ABRC).

The *Arabidopsis thaliana Resource Center for Genomics* from the Versailles Center at INRA have currently three collections available for the scientific community: a) a collection of more than 500 ecotypes; b) a set of independent T-DNA insertion mutants in Wassilewskija background (FLAG mutants); and c) several recombinant

inbred line populations. These resources can be directly ordered from an autonomous INRA server.

Other plant material can also be found in smaller collections. The GABI-Kat lines comprise confirmed T-DNA insertion mutants that were subsequently characterized by generating flanking sequence tags. Currently, these lines are being donated to NASC in a process that has already been initiated. The *RIKEN BioResource Center* (RIKEN BRC) also preserves and distributes T-DNA/transposon mutant seeds and cDNAs. Plant cell cultures are also available for order in this stock center. Finally, the LEHLE SEEDS collection provides several types of mutagenized seeds, produced either with EMS, fast neutrons or gamma-rays.

Gene knockout mutants and knockdown lines

A conclusive evidence of biological function cannot be predicted simply by genome sequence or transcriptional regulation analysis. The experimental evidence is still needed. Insertion mutant analysis is considered to be an improved and reliable way to give essential clues to the function of the disrupted gene. In the process of generating tools for reverse genetics research, several collections of mutant lines were made available to the scientific community. Tools for searching the localization and sequences of the mapped insertion sites in the majority of the assembled mutant lines are freely available in ATIDB (Pan *et al.* 2003) and SIGnAL T-DNA Express (Alonso *et al.* 2003). Using these resources, biologists can select the mutant line of interest and subsequently order the corresponding seeds stock using the suitable links to stock centers. As mutant seeds are often provided as a genotypically heterogeneous population, specific selection of homozygous mutant lines has to be achieved prior to subsequent functional analysis. In order to facilitate this task, the SIGnAL T-DNA Express platform also allows the use of an efficient tool for primers designing. Using the SIGnAL T-DNA Primer Design website, PCR primers sequence, PCR conditions and expected fragment size could be determined. As described in the T-DNA Primer manual, genotypic screening of mutant seeds can be achieved by analysis of the number and dimension of amplified fragments. The T-DNA insertion could then be detected in two, just one or none of the homologous chromosomes.

Recently, RNAi has been gradually introduced as an alternative technique to disturb gene expression, particularly in genes lacking an insertion mutation or in embryo-lethal mutant genes. RNAi technology takes advantage on direct targeting of a specific gene or genes to produce lines with variable levels of gene silencing, using the same RNAi hairpin construct. The timing and extent of the gene silencing can also be controlled, so that genes that are essential will only be silenced at chosen stages of growth or in chosen plant tissues (Matthew 2004). The AGRIKOLA consortium developed several RNAi constructs for the large-scale systematic RNAi silencing of *Arabidopsis* genes (Hilson *et al.* 2004). The gene-specific sequence tags (GST) used for producing such RNAi constructs had been designed by the CATMA project, which aimed to design and produce high quality GSTs covering most of the *Arabidopsis* genome. This kind of targeted mutagenesis will complement the numerous insertion mutant collections and also generate allelic series that represent different levels of silencing. Furthermore, CATMA database displays the designed GSTs and AGRIKOLA resource offers the possibility to order the produced clones or even the corresponding silenced lines seeds.

Additional functional information

Other accessible bioinformatics resources can be extremely useful in reaching higher informative levels that could assist in the elucidation of gene function. A complementary or alternative approach for functional discovery is the analysis of co-regulated genes. The function of certain genes related to a particular pathway could be inferred by their co-expression to already characterized genes of a known pathway (reviewed by Saito *et al.* 2007). A successful example of the potential of this strategy was the identification of genes required for cellulose synthesis (Persson *et al.* 2005) and brassinosteroid related genes (Lisso *et al.* 2005). To assist the deduction of gene function based on co-responsive genes, researchers can explore ATTED-II resource that uses the microarray data of 58 publicly available experimental series (Obayashi *et al.* 2009). Another co-expression database, the *A. thaliana Co-Response Database* included in the *Comprehensive Systems-Biology Database* (AthCoR@CSB.DB), was developed for the same purpose (Steinhauser *et al.* 2004). Additional information on promoter sequences and putative *cis*-regulatory elements can be predicted in ATTED-II

and AGRIS resources (Davuluri *et al.* 2003; Palaniswamy *et al.* 2006). This last server is composed by two different databases functioning in *tandem* (AtcisDB for *cis*-elements and AtTFDB for transcription factors), both providing the essential software tools on *A. thaliana* transcription factors and their putative binding sites for all genes. This resource is of great help for identifying the associated transcriptional regulatory networks.

A great number of other databases and tools are currently available for supplementary functional data, and this number is growing day-by-day. For example, studies on plant membrane proteins can make use of ARAMEMNON (Schwacke *et al.* 2003). This database includes data concerning protein description, prediction for transmembrane spanning proteins and membrane-anchored proteins and prediction of their subcellular location (chloroplast, mitochondrion or secretory pathways). Also, in a pilot project that is still in progress, the subcellular localization of various *Arabidopsis* gene products is being studied by the AZTEC project (Li *et al.* 2006). By using a newly developed high-throughput fluorescent tagging of proteins, the information of subcellular localization of all *Arabidopsis* proteins will be soon determined.

Recently, in order to improve the number of functionally assigned proteins and overcome the laborious and time consuming experimental validation, emerging areas of bioinformatics are being developed. New rules for the prediction of gene function in *Arabidopsis* from diverse data sources have been created based on attribute-value data mining/machine learning methods (Clare *et al.* 2006).

Gene expression data

The amount of data on gene expression that had been produced by numerous microarray experiments worldwide, established the need to generate databases to accumulate this information. Currently, NASCArrays (Craigon *et al.* 2004) and ArrayExpress (Parkinson *et al.* 2007) are the major repositories for the created expression data. NASCArrays data was attained by using two GeneChips from Affymetrix. The smaller and older GeneChip design represents ~8,000 genes (Zhu and Wang 2000), while the recent ATH1 GeneChip design (~23,750 genes) covers almost all the *Arabidopsis* genome (Redman *et al.* 2004). NASCArrays database provides data on

transcript levels of *A. thaliana* major organs, development stages as well as in response to diverse stimuli: chemical, abiotic and biotic. ArrayExpress stores functional genomic data in two independent components specialized for distinct purposes: the *ArrayExpress Repository* that archives microarray data and the *ArrayExpress Data Warehouse*, which is a database of gene expression profiles. The data incorporated both in NASCArrays and ArrayExpress are MIAME-compliant, which means that data from diverse experiments can be easily interpreted and statistically comparable by the use of standard descriptions of experimental conditions (Brazma *et al.* 2001). Data submitted into the databases is easily accessible by simple keyword searching and data mining tools. As microarray data are freely available for download, these web-based tools become a powerful resource for gene discovery and functional research.

Simultaneous to the creation of expression databases, a number of associated on-line tools were developed to identify experiments of interest, as well as for gene expression profile analysis. In this context, *Genevestigator* appears as a complete suite of user-friendly tools incorporating a reference expression database and meta-analysis system, which summarizes information from hundreds of microarray experiments into easily interpretable results (Zimmermann *et al.* 2004; Zimmermann *et al.* 2005). An updated Java-based version of *Genevestigator* provides improved querying features and includes the following tool sets: Meta-profile analysis, Biomarker search, Clustering analysis and Pathway projector (Hruz *et al.* 2008). The Meta-profile analysis tool set facilitates the visualization of transcriptional differences in the context of plant organs and tissues, growth stages and stressing conditions. Results can be displayed for one particular gene or entire gene families. Additionally to these features, the *Botany Array Resource* (BAR) provides an extra set of tools, such as hierarchical clustering, the ability to automatically calculate the averages and reasons of treatment against control samples and sophisticated tools for detection of co-expressed genes and *cis*-elements on the promoter of a specific gene (Toufighi *et al.* 2005). Also, the possibility to analyze large data sets or predict subcellular localizations with quite easy-understandable representations is available in BAR using eFP Browser (Winter *et al.* 2007). A more specific database directed particularly for *Arabidopsis* root analysis was also created based on spatial gene expression experiments. The *Arabidopsis Gene Expression Database* (AREX) offers the possibility to visualize the expression pattern of

a certain gene by performing an *in situ* digital analysis predicted from microarray data (Birnbaum *et al.* 2003). This tool also allows the detection of genes displaying the same expression pattern.

The application of all these tools for transcriptional studies avoids the strict demands of statistical and software analysis, commonly associated with microarrays. Therefore, the use of such tools becomes an attractive method for setting off a reverse genetics approach (Rensink and Buell 2005). However, never questioning the handful application of gene expression analysis in scientific research, functional inferences based only in this sort of data must be carefully taken. As reported in other model organisms, genes selected by expression alterations upon microarray analysis using a particular stress situation not always have their transcription regulated by that kind of stressful condition (Giaever *et al.* 2002).

SEARCHING FOR GENES OF INTEREST - THE PRACTICAL CASE OF HEAT STRESS DETERMINANT GENES

The use of DNA microarrays analysis has progressively become the most accepted method to detect, at the whole genome level, any transcriptional differences in response to altered conditions. As reviewed by Wullschleger and Difazio (2003), several research projects reported the successful use of gene expression arrays to detect qualitative differences resulting from exposure of different plant species to manipulated temperature, water content and aluminium levels. At the moment, the use of gene expression microarrays analysis is frequently found in the literature. In *A. thaliana* model plant, the elucidation of stress responses at a genomic level has been greatly accomplished by expression profile analysis. Particularly, several genes playing a role in wounding, cold, salt, drought and heat stresses were identified by transcriptional analysis (Cheong *et al.* 2002; Rizhsky *et al.* 2004; Oono *et al.* 2006; reviewed by Sreenivasulu *et al.* 2007; Swindell *et al.* 2007).

In this work, we propose a straightforward strategy, supported by reverse genetics principles, for the identification of determinant genes for thermotolerance in *A. thaliana*. By using the expression data acquired in publicly available microarray experiments, the identification of one or more putative heat stress determinant genes would be the preliminary step of the proposed strategy. The success of this strategy will be accomplished after establishing a heat stress-associated phenotype in loss-of-function mutants on selected genes (Chapter 5).

Identification of putative heat determinants in publicly available microarrays analysis

The expression levels of ~23,750 genes in heat-stressed *Arabidopsis* plants and cell suspensions were analysed using Affymetrix microarray gene expression profiles. Data access was provided by NASC International Affymetrix service by downloading significant experiment data from NASCArrays database. As the selection of putative determinant genes for thermotolerance was pretended, the *heat stress time course* experiment performed in shoots, roots and cell suspensions of *A. thaliana* was considered. This experiment is included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007) and made use of the ATH1 GeneChip design. After 3 h of light treatment, 16-day-old seedlings were subjected to a heat stress of 38°C for 3 h, in an incubator.

Samples were taken at 0.25, 0.5, 1 and 3 h during heat stress and at 4, 6, 12 and 24 h during recovery at 25°C. Sampled shoots and roots were prepared separately. A similar treatment was performed for 6-day-old cell suspensions using the same sampling intervals. In this experiment, heat stress was imposed by immersion of culture flasks in a water bath. Controls (shoots, roots and cell suspensions) were prepared in parallel using the temperature of 25°C. Downloaded data included the expression signal and complete annotation of each gene, without data compression.

Statistical data treatment was performed calculating the reason between the expression of a certain gene in a particular condition and the expression in the corresponding control (relative expression). However, for selection of potential thermotolerance determinants, genes were also sorted based on absolute expression, determined by the signal intensity of gene expression in pixel counts. Gene assortment was performed when relative expression values were above two and absolute expression values were higher than 500 pixels, as described by Moseiko *et al.* (2002). This analysis was performed for each sampling time and the number of up-regulated genes assembling these attributes was plotted against heat-treatment and recovery period (Figure 3.3). Considering the high number of genes with two-fold enhanced expression in heat-stressed cell suspensions, the ten-fold relative expression threshold was also considered.

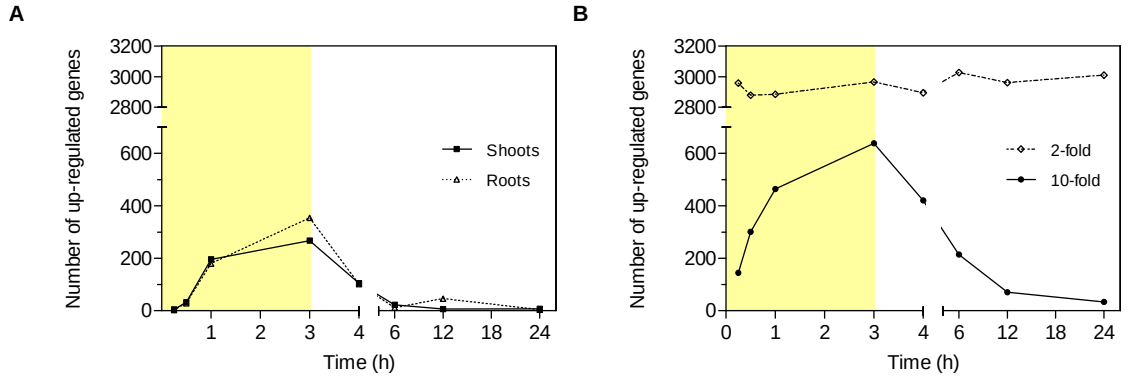


Figure 3.3. Number of up-regulated genes after heat stress imposition on *A. thaliana* shoots and roots (A) and cell suspensions (B). The data was obtained from the *heat stress time course* experiment, included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007). After 3 h of light treatment, 16-day-old plants or 6-day-old cell suspensions were subjected to a heat stress of 38°C for 3 h (highlighted in yellow) and were then allowed to recover at 25°C. Only those genes with absolute expression values above 500 pixels and relative expression values above two-fold (or ten-fold for cell suspensions) were considered.

In heat-stressed plants and cell suspensions, the number of up-regulated genes rapidly increased just after stress onset and continued to rise till the end of stress imposition (3 h). During recovery at 25°C, the number of genes displaying relative expression values above two rapidly dropped off. This variation in up-regulated genes number was observed for both shoots and roots when the relative expression level was set for two-fold. However, the same variation in up-regulated genes was only observed for cell suspensions after considering genes with expression increases above ten-fold.

Besides avoiding an extensive data analysis, the used approach provided a great number of genes with strong putative involvement on thermotolerance. A comparative analysis of differentially expressed genes was performed by crossing-over the results obtained at different times. The expression profile of each gene allowed to distinguish seven different classes of gene response (Figure 3.4). For both shoots and roots, a

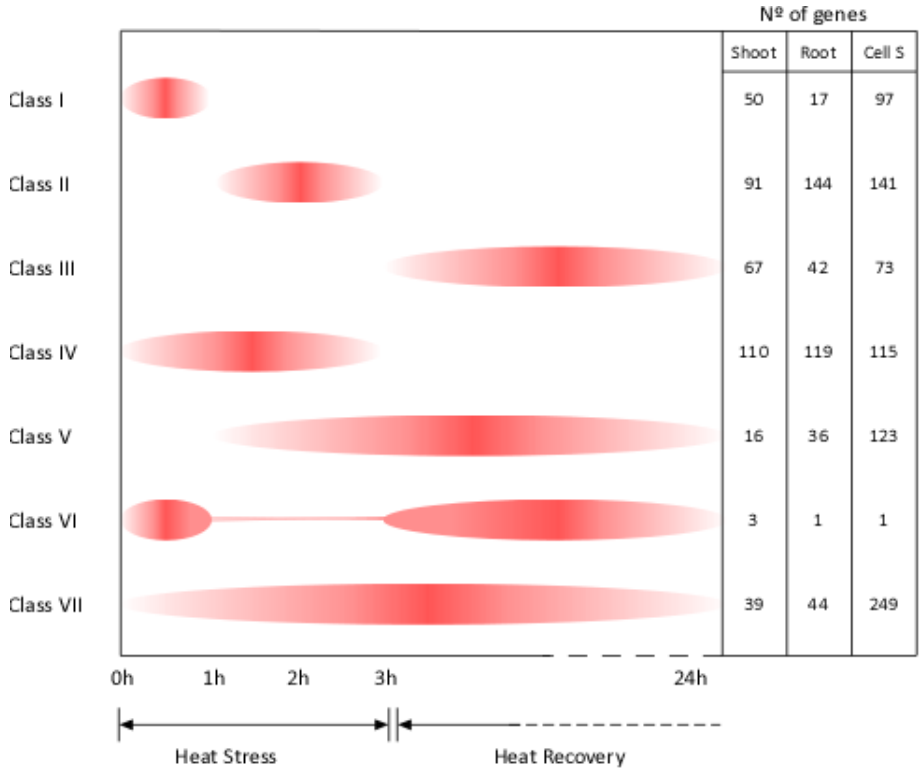


Figure 3.4. Different classes of heat stress responsive genes displayed by *A. thaliana* shoots, roots and cell suspensions. The expression profiles of up-regulated genes during the *heat stress time course* experiment were analysed and genes with similar profiles were grouped together within classes. In this experiment, included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007), after 3 h of light treatment, 16-day-old plants or 6-day-old cell suspensions were subjected to a heat stress of 38°C for 3 h and were then allowed to recover at 25°C. Only those genes with absolute expression values above 500 pixels and relative expression values above two-fold (shoots and roots) or ten-fold (cell suspensions) were considered.

similar response profile was found. A small number of genes (50 in shoots and 17 in roots) was detected exclusively during the first hour of stress imposition (class I). In shoots and roots samples, the major number of up-regulated genes was found during heat stress imposition. These genes include those only up-regulated late after stress imposition (class II) or during the overall period of stress (class IV). In contrast with heat stress induced genes, during the recovery period a smaller number of genes were up-regulated. The majority was up-regulated after the end of heat treatment (class III), but few had already been up-regulated late during stress treatment (class V). Also, a number of genes displaying enhanced expression levels just after the heat imposition maintained the high expression levels during the all time-course of the experiment (class VII). The strange profile of class VI genes was only depicted by an irrelevant number of genes.

The physiological response of plants to heat stress depends on the initial perception of temperature alteration to trigger the subsequent transduction signals. These signals would trigger downstream signaling and transcriptional events, which activate mechanisms to recover cellular homeostasis, often by modification of gene expression (reviewed by Wahid *et al.* 2007). Under heat stress two different groups of genes are induced: those responsible for regulation of gene expression and signal transduction pathways and those directly related with protection against stress (reviewed by Kaur and Gupta 2005). In the first group are included those genes encoding transcription factors, protein kinases and phosphoinositide metabolism enzymes, which are triggered after the initial recognition of stress. These early response genes are normally induced quickly and transiently and function to activate the delayed response genes presenting a more sustained expression. In this second group of heat-induced genes are included those encoding osmoprotectant enzymes, antifreeze proteins, chaperones and scavenging enzymes (reviewed by Kaur and Gupta 2005). The time frame induction of a particular gene can thus reflect the role of the corresponding gene product in the response pathway. Accordingly, the early response genes that were activated just after imposition of heat stress and became inactive 1-3 h after treatment (class I genes) might encode for signaling or regulatory components, such as protein kinases and transcription factors. Analysis of class I genes revealed a percentage of approximately 18-22% genes implicated in the transcriptional

regulatory networks in all biological samples (~25-30% were unknown genes). These genes would probably be responsible for the initiation of stress response. The delayed response genes activated later during stress imposition (class II and V genes) would probably encode effector proteins, such as proteins involved in protection and repair of protein and membrane damage caused by stress and in re-establishing the cellular homeostasis. Although they have been up-regulated during the first hour of stress imposition, class IV and VII genes might also encode for effector proteins since they present a more sustained expression profile during stress treatment. Altogether, these genes seem to be responsible for thermotolerance. Those genes highly expressed during recovery (class III) would probably be late effector proteins. As protein products of early response genes could regulate the expression of delayed response genes, a cascade of gene regulation has already been suggested (reviewed by Kaur and Gupta 2005). Accordingly, the small set of genes found in class VI reflect the transient nature of the heat shock response.

When compared to the general gene expression profile depicted by shoots and roots, cell suspensions present distinctive features. The need for a ten-fold threshold in relative expression values to detect a similar profile as the one observed in shoots and roots suggests stronger gene induction in cell suspensions subjected to heat stress. During the first hour of stress imposition, a much higher number of genes were induced (classes I and VII), much of them presenting a sustained expression during the overall experiment period (class VII). This stronger response would be probably due to the higher sensibility of suspended cells in sensing temperature heat stress when compared to cells integrated into a tissue (shoots and roots). Additionally, cell suspensions were treated by immersion into a water bath, which enhances heat transfer to the cells, in opposition to the whole plant treatment applied in an incubator.

In order to identify the genes that are more associated to the thermotolerance, the genes that were up-regulated after 3 h of heat imposition, in all the biological samples (shoots, roots and cell suspensions) were selected (Figure 3.5). Prior to this analysis, those genes included in mitochondrial or plastidial genomes were discarded (ten genes). The 137 overexpressed genes (Table 3.2) displaying those features included genes from classes II, IV, V and VII.

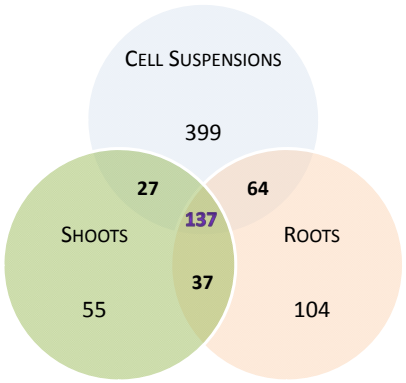


Figure 3.5. Up-regulated genes after 3 h of heat stress treatment on *A. thaliana* shoots, roots and cell suspensions. The data was obtained from the *heat stress time course* experiment, included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007). After 3 h of light treatment, 16-day-old plants or 6-day-old cell suspensions were subjected to a heat stress of 38°C for 3 h. Only those genes with absolute expression values above 500 pixels and relative expression values above two-fold (shoots and roots) or ten-fold (cell suspensions) after 3 h of stress treatment were considered.

Expression patterns in different contexts supported the selection of the most determinant genes

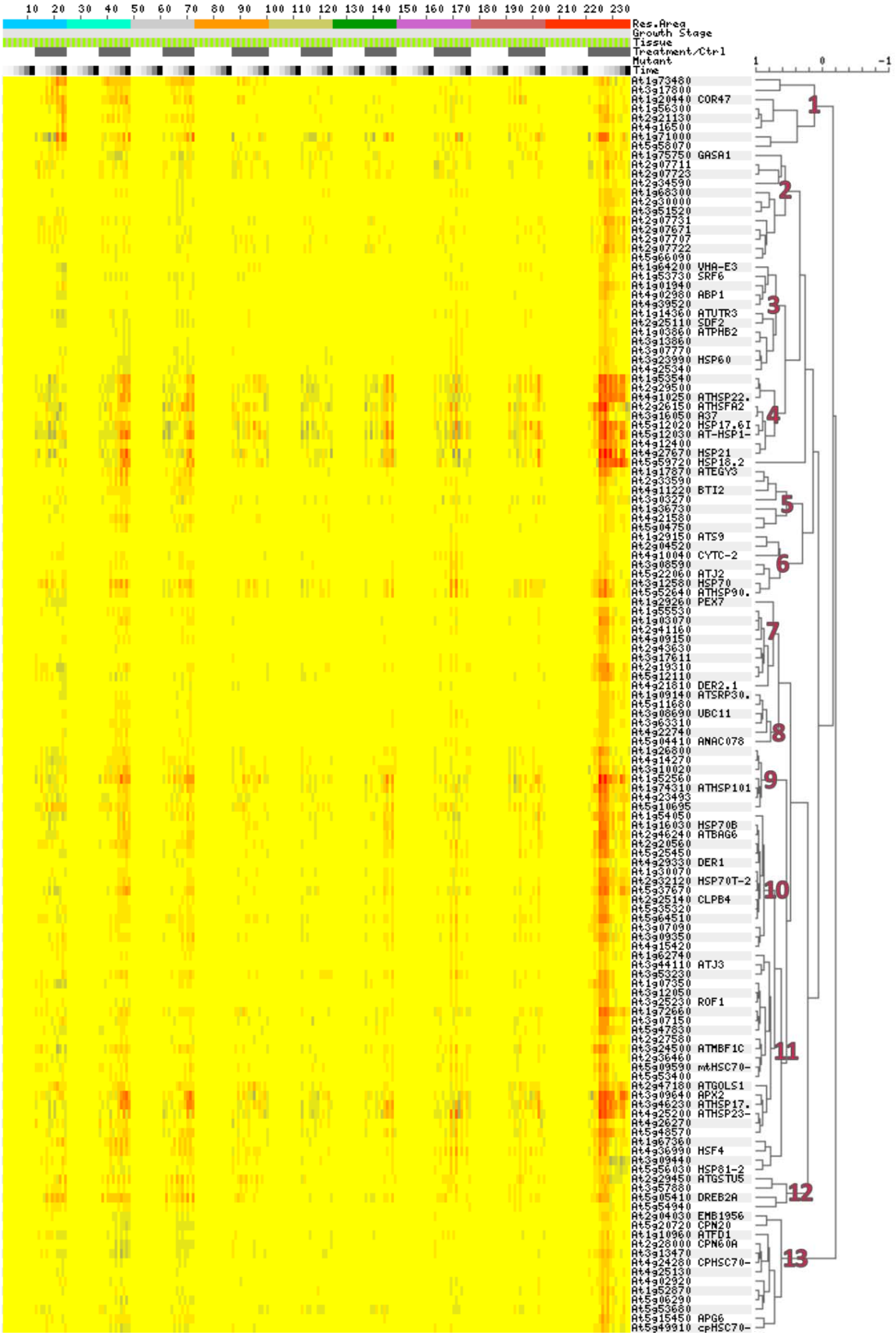
Previous gene expression analysis retrieved an extensive list of putative thermotolerance-associated genes (137 entries, Table 3.2). The expression pattern analysis of these heat-stressed genes in response to diverse stimulus allowed the detection of the most specific heat responsive transcripts. Using the Expression Browser tool provided by BAR, an electronic Northern of these genes under several abiotic stresses (cold, osmotic, salt, drought, genotoxic, oxidative, UV-B, wounding and heat) was performed (Figure 3.6). An automatic clustering of transcripts according to the similarities of their expression patterns was performed by activating the program Data Metaformatter. The data was clustered hierarchically using an uncentered correlation similarity metric (Pearson's correlation coefficient) and average linkage clustering. Thirteen different clusters of genes were identified (Figure 3.6), five of which presented apparent higher heat specificity (clusters 2, 3, 7, 8 and 13). The gene clusters 1, 4 and 11 were the less heat-specific since higher expression levels were also found in the other types of abiotic stimulus.

Further analysis involved the evaluation of the gene expression levels in response to additional stimulus, other than abiotic, which was performed individually for each gene cluster previously identified (Figure 3.7). This analysis was performed using the Stimulus tool, included in the Meta-profile analysis tool set of the web-based

Table 3.2 AGI numbers of the 137 up-regulated genes in either *A. thaliana* shoots, roots or cell suspensions after 3 h of heat stress treatment as represented in Figure 3.5. Those genes whose expression was not so strongly heat-specific are highlighted in grey (results from Figures 3.6 and 3.7).

AGI				
Chr 1	**Chr 2**	**Chr 3**	**Chr 4**	**Chr 5**
At1g01940	At2g04030	At3g03270	At4g02920	At5g04410
At1g03070	At2g04520	At3g07090	At4g02980	At5g04750
At1g03860	At2g07671	At3g07150	At4g09150	At5g05410
At1g07350	At2g07707	At3g07770	At4g10040	At5g06290
At1g09140	At2g07711	At3g08590	At4g10250	At5g09590
At1g10960	At2g07722	At3g08690	At4g11220	At5g10695
At1g14360	At2g07723	At3g09350	At4g12400	At5g11680
At1g16030	At2g07731	At3g09440	At4g14270	At5g12020
At1g17870	At2g19310	At3g09640	At4g15420	At5g12030
At1g20440	At2g20560	At3g10020	At4g16500	At5g12110
At1g26800	At2g21130	At3g12050	At4g21320	At5g15450
At1g29150	At2g25110	At3g12580	At4g21580	At5g20720
At1g29260	At2g25140	At3g13470	At4g21810	At5g22060
At1g30070	At2g26150	At3g13860	At4g22740	At5g25450
At1g36730	At2g27580	At3g16050	At4g23493	At5g35320
At1g52560	At2g28000	At3g17611	At4g24280	At5g37670
At1g52870	At2g29450	At3g17800	At4g25130	At5g47830
At1g53540	At2g29500	At3g23990	At4g25200	At5g48570
At1g53730	At2g30000	At3g24100	At4g25340	At5g49910
At1g54050	At2g32120	At3g24500	At4g26270	At5g52640
At1g55530	At2g33590	At3g25230	At4g27670	At5g53400
At1g56300	At2g34590	At3g44110	At4g29330	At5g53680
At1g62740	At2g36460	At3g46230	At4g36990	At5g54940
At1g64200	At2g41160	At3g51520	At4g39520	At5g56030
At1g67360	At2g43630	At3g53230		At5g58070
At1g68300	At2g46240	At3g57880		At5g59720
At1g71000	At2g47180	At3g63310		At5g64510
At1g72660				At5g66090
At1g73480				
At1g74310				
At1g75750				

AGI: *A. thaliana* genome initiative number; Chr: chromosome


10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230
Res.Area
Growth Stage
Tissue
Treatment/Ctrl
Mutant
Time
1 0 -1
At1g73480
At3g17800
At1g20440 COR47
At1g56300
At2g21130
At4g16500
At1g71000
At5g58070
At1g75750 GASA1
At2g07711
At2g07723
At2g34590
At1g68300
At2g30000
At5g51520
At2g07731
At2g07671
At2g07707
At2g07722
At5g66090
At1g64200 VHA-E3
At1g53730 SRF6
At1g01940
At4g02980 ABP1
At4g39520
At1g14360 ATUTR3
At2g25110 SDF2
At1g03860 ATPHB2
At3g13860
At3g07770
At3g23990 HSP60
At4g25340
At1g53540
At2g29500
At4g10250 ATHSP22.
At2g26150 ATHSFA2
At3g16050 A37
At5g12020 HSP17.6I
At5g12030 AT-HSP1-
At4g12400
At4g27670 HSP21
At5g59720 HSP18.2
At1g17870 ATEGY3
At2g33590
At4g11220 BTI2
At3g03270
At1g36750
At4g21580
At5g04750
At1g29150 ATS9
At2g04520
At4g10040 CYTC-2
At3g08590
At5g22060 ATJ2
At3g12580 HSP70
At5g52640 ATHSP90.
At1g29260 PEX7
At1g55530
At1g03070
At2g41160
At4g09150
At2g43630
At3g17810
At2g19310
At5g12110
At4g21810 DER2.1
At1g09140 ATSRP30.
At5g11660
At3g08690 UBC11
At3g63310
At4g22740
At5g04410 ANAC078
At1g26800
At4g14270
At3g10020
At1g52560
At1g74310 ATHSP101
At4g23493
At5g10695
At1g54050
At1g16030 HSP70B
At2g46240 ATBAG6
At2g20560
At5g25450
At4g29330 DER1
At1g30070
At2g32120 HSP70T-2
At5g37670
At2g25140 CLPB4
At5g35320
At5g64510
At3g07090
At3g09350
At4g15420
At1g62740
At3g44110 ATJ3
At3g53230
At1g07350
At3g12050
At3g25230 ROF1
At1g72660
At3g07150
At5g47830
At2g27580
At3g24500 ATMBF1C
At2g36460
At5g09590 mtHSC70-
At5g53400
At2g47180 ATGOLS1
At3g09640 APX2
At3g46230 ATHSP17.
At4g25200 ATHSP23-
At4g26270
At5g48570
At1g67360
At4g36990 HSF4
At5g09440
At5g56030 HSP81-2
At2g29450 ATGSTU5
At3g57860
At5g05410 DREB2A
At5g54940
At2g04030 EMB1956
At5g20720 CPN20
At1g10960 ATFD1
At2g28000 CPN60A
At3g13470
At4g24280 CPHSC70-
At4g25130
At4g02920
At1g52870
At5g06290
At5g53600
At5g15450 APG6
At5g49910 cpHSC70-
1
2
3
4
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9
10
11
12
13

Genevestigator software. This evaluation enabled the sorting of the genes displaying higher specificity towards heat stress, by manually discarding the genes whose responsiveness was common among stresses (highlighted in grey in Table 3.2). At the end, 43 genes were selected as putative determinants for thermotolerance in *A. thaliana* (Table 3.3). The expression profile of these genes in response to several external stimuli is displayed in Figure 3.8, while the results concerning the other non-selected genes are displayed in Annex I.

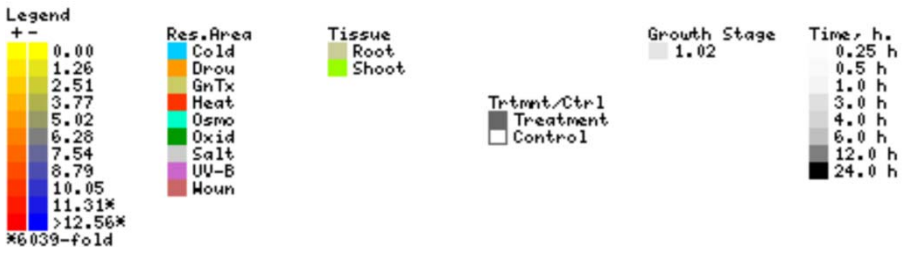
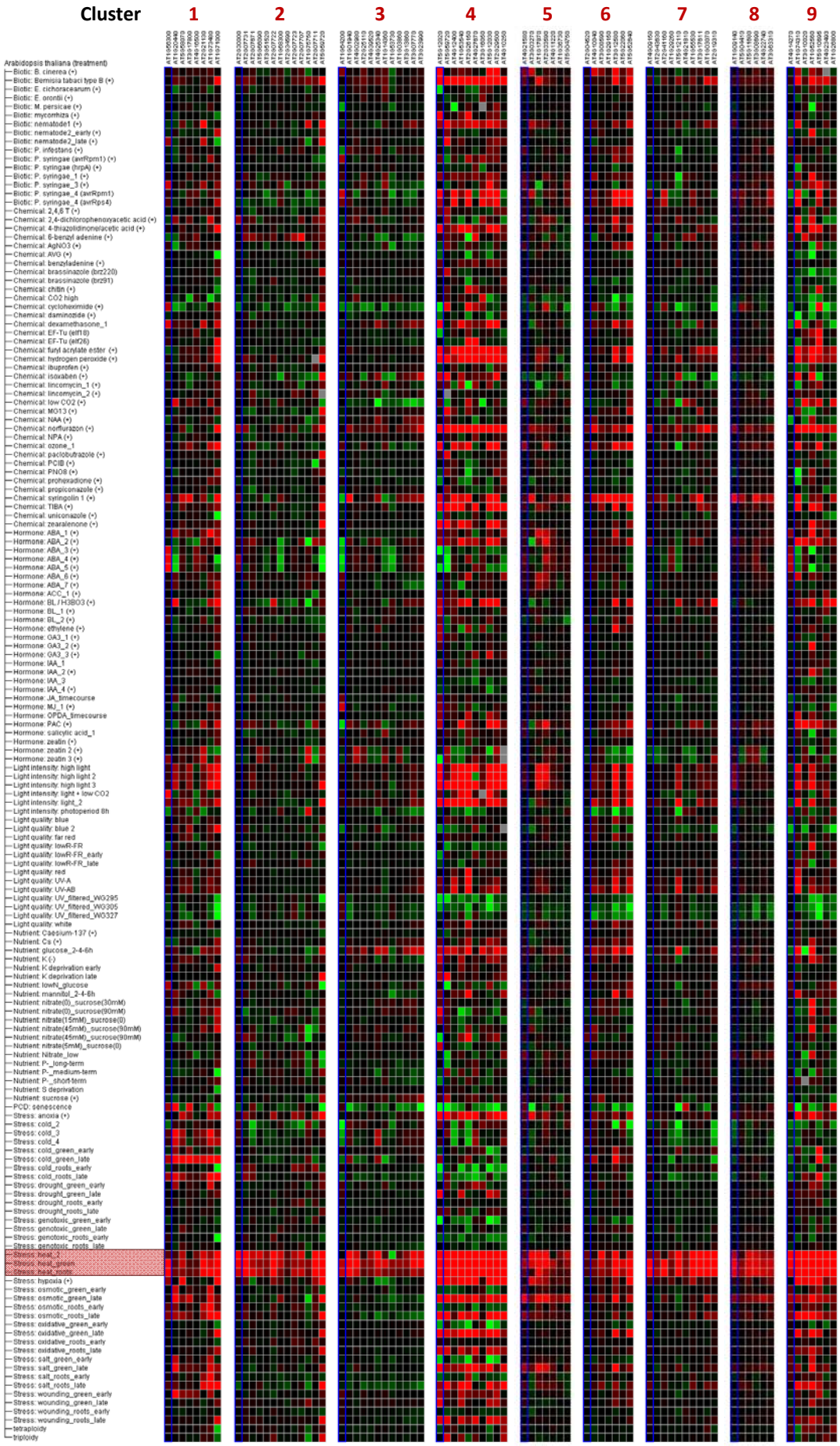
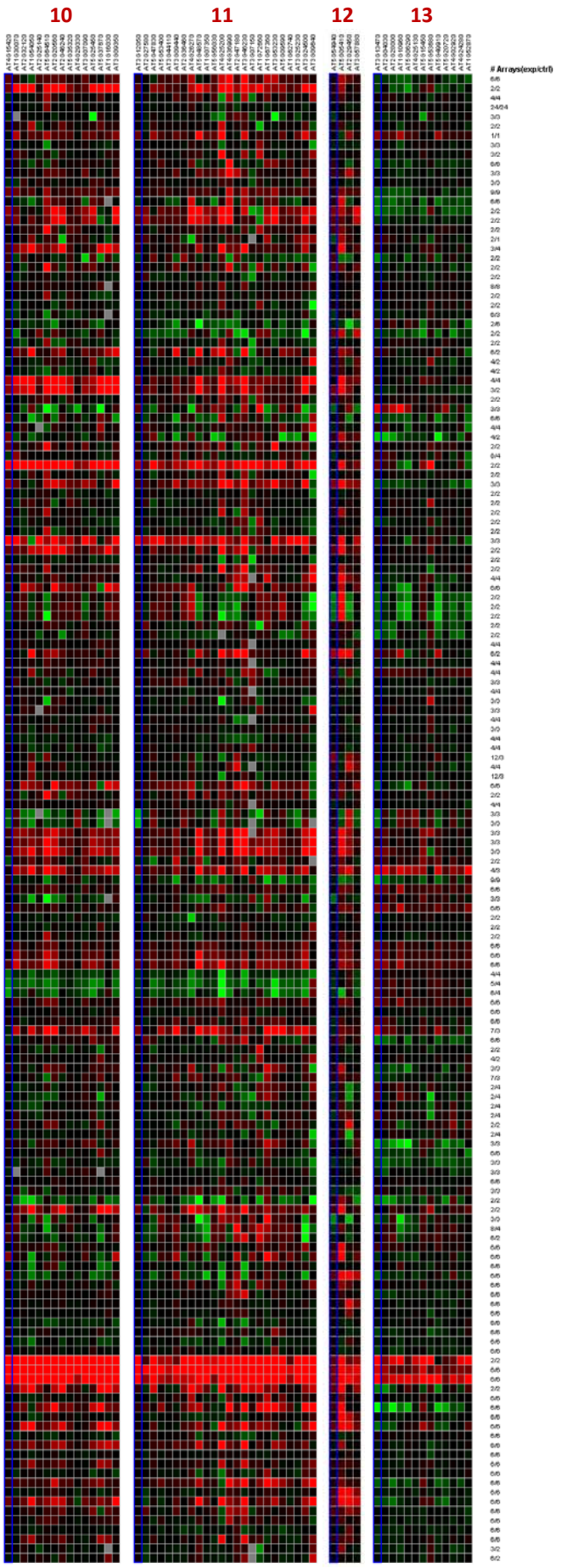


Figure 3.6. Expression profile of the 137 *A. thaliana* genes (up-regulated in either shoots, roots or cell suspensions after 3 h of heat stress treatment) in response to different abiotic factors, according to the Expression Browser (electronic Northern tool) of BAR. AGI number and cluster tree with co-regulated genes grouped together are displayed on the right. Numbers indicate the considered gene clusters. Expression levels, experiments, tissue types, treatments and time sampling are identified in colors as depicted above.


Cluster
1
2
3
4
5
6
7
8
9
Arabidopsis thaliana (treatment)
Biotic: B. cinerea (+)
Biotic: Bemisia tabaci type B (+)
Biotic: E. cichoracearum (+)
Biotic: E. orontii (+)
Biotic: M. persicae (+)
Biotic: mycorrhiza (+)
Biotic: nematode1 (+)
Biotic: nematode2_early (+)
Biotic: nematode2_late (+)
Biotic: P. infestans (+)
Biotic: P. syringae (avrRpm1) (+)
Biotic: P. syringae (hrpA) (+)
Biotic: P. syringae_1 (+)
Biotic: P. syringae_3 (+)
Biotic: P. syringae_4 (avrRpm1)
Biotic: P. syringae_4 (avrRps4)
Chemical: 2,4,6 T (+)
Chemical: 2,4-dichlorophenoxyacetic acid (+)
Chemical: 4-thiazolidinonearacetic acid (+)
Chemical: 6-benzyl adenine (+)
Chemical: AgNO3 (+)
Chemical: AVG (+)
Chemical: benzyladenine (+)
Chemical: brassinazole (brz220)
Chemical: brassinazole (brz91)
Chemical: chitin (+)
Chemical: CO2 high
Chemical: cycloheximide (+)
Chemical: daminozide (+)
Chemical: dexamethasone_1
Chemical: EF-Tu (elf18)
Chemical: EF-Tu (elf26)
Chemical: furyl acrylate ester (+)
Chemical: hydrogen peroxide (+)
Chemical: ibuprofen (+)
Chemical: isoxaben (+)
Chemical: lincomycin_1 (+)
Chemical: lincomycin_2 (+)
Chemical: low CO2 (+)
Chemical: MG13 (+)
Chemical: NAA (+)
Chemical: norflurazon (+)
Chemical: NPA (+)
Chemical: ozone_1
Chemical: paclobutrazole (+)
Chemical: PCIB (+)
Chemical: PNO8 (+)
Chemical: prohexadione (+)
Chemical: propiconazole (+)
Chemical: syringolin 1 (+)
Chemical: TIBA (+)
Chemical: uniconazole (+)
Chemical: zearalenone (+)
Hormone: ABA_1 (+)
Hormone: ABA_2 (+)
Hormone: ABA_3 (+)
Hormone: ABA_4 (+)
Hormone: ABA_5 (+)
Hormone: ABA_6 (+)
Hormone: ABA_7 (+)
Hormone: ACC_1 (+)
Hormone: BL / H3BO3 (+)
Hormone: BL_1 (+)
Hormone: BL_2 (+)
Hormone: ethylene (+)
Hormone: GA3_1 (+)
Hormone: GA3_2 (+)
Hormone: GA3_3 (+)
Hormone: IAA_1
Hormone: IAA_2 (+)
Hormone: IAA_3
Hormone: IAA_4 (+)
Hormone: JA_timecourse
Hormone: MJ_1 (+)
Hormone: OPDA_timecourse
Hormone: PAC (+)
Hormone: salicylic acid_1
Hormone: zeatin (+)
Hormone: zeatin 2 (+)
Hormone: zeatin 3 (+)
Light intensity: high light
Light intensity: high light 2
Light intensity: high light 3
Light intensity: light + low CO2
Light intensity: light_2
Light intensity: photoperiod 8h
Light quality: blue
Light quality: blue 2
Light quality: far red
Light quality: lowR-FR
Light quality: lowR-FR_early
Light quality: lowR-FR_late
Light quality: red
Light quality: UV-A
Light quality: UV-AB
Light quality: UV_filtered_WG295
Light quality: UV_filtered_WG305
Light quality: UV_filtered_WG327
Light quality: white
Nutrient: Caesium-137 (+)
Nutrient: Cs (+)
Nutrient: glucose_2-4-6h
Nutrient: K (-)
Nutrient: K deprivation early
Nutrient: K deprivation late
Nutrient: lowN_glucose
Nutrient: mannitol_2-4-6h
Nutrient: nitrate(0)_sucrose(30mM)
Nutrient: nitrate(0)_sucrose(90mM)
Nutrient: nitrate(15mM)_sucrose(0)
Nutrient: nitrate(45mM)_sucrose(90mM)
Nutrient: nitrate(45mM)_sucrose(90mM)
Nutrient: nitrate(5mM)_sucrose(0)
Nutrient: Nitrate_low
Nutrient: P-_long-term
Nutrient: P-_medium-term
Nutrient: P-_short-term
Nutrient: S deprivation
Nutrient: sucrose (+)
PCD: senescence
Stress: anoxia (+)
Stress: cold_2
Stress: cold_3
Stress: cold_4
Stress: cold_green_early
Stress: cold_green_late
Stress: cold_roots_early
Stress: cold_roots_late
Stress: drought_green_early
Stress: drought_green_late
Stress: drought_roots_early
Stress: drought_roots_late
Stress: genotoxic_green_early
Stress: genotoxic_green_late
Stress: genotoxic_roots_early
Stress: genotoxic_roots_late
Stress: heat_3
Stress: heat_green
Stress: heat_roots
Stress: hypoxia (+)
Stress: osmotic_green_early
Stress: osmotic_green_late
Stress: osmotic_roots_early
Stress: osmotic_roots_late
Stress: oxidative_green_early
Stress: oxidative_green_late
Stress: oxidative_roots_early
Stress: oxidative_roots_late
Stress: salt_green_early
Stress: salt_green_late
Stress: salt_roots_early
Stress: salt_roots_late
Stress: wounding_green_early
Stress: wounding_green_late
Stress: wounding_roots_early
Stress: wounding_roots_late
tetraploidy
triploidy



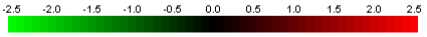


Figure 3.7. Expression profile of *A. thaliana* genes (up-regulated in either shoots, roots or cell suspensions after 3 h of heat stress treatment) in response to different external biotic or abiotic stimuli, according to the Meta-profile analysis (Stimulus tool) of *Genevestigator*. Results are given as heat maps in red/green coding. Red indicates that the signal intensity of the treatment is higher than signal intensity of the corresponding control, and green means the opposite. This analysis was performed for the 137 genes grouped in 13 clusters, according to the electronic Northern presented in Figure 3.6. Co-regulation cluster and AGI numbers are displayed on the top. Treatments are indicated on the left, where heat treatments are highlighted in red.

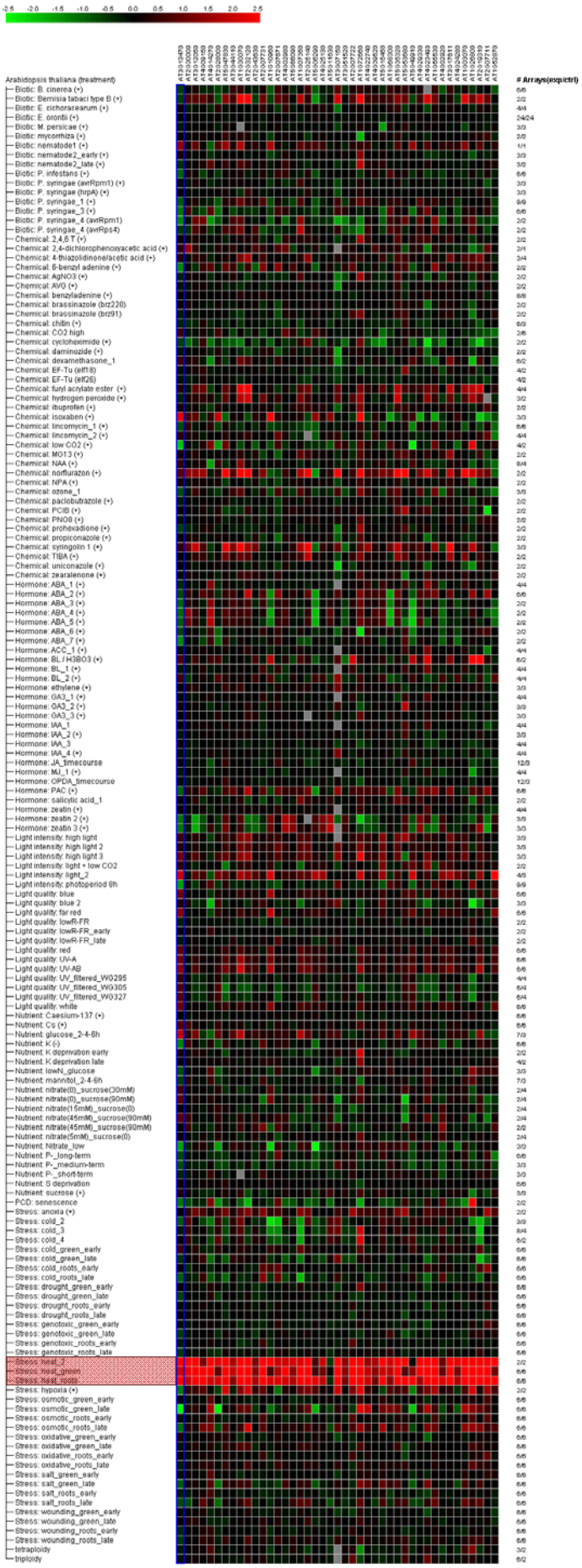


Figure 3.8. Expression profile of the 43 *A. thaliana* most heat-specific selected genes, in response to different external biotic or abiotic stimuli according to the Meta-profile analysis (Stimulus tool) of *Genevestigator*. Results are given as heat maps in red/green coding. Red indicates that the signal intensity of the treatment is higher than signal intensity of the corresponding control, and green means the opposite. AGI number is displayed on the top. Treatments are indicated on the left, where heat treatments are highlighted in red.

In order to get more information about selected genes, the Anatomy and Development tools (Meta-profile analysis tool set, *Genevestigator*) were used for determining on which organs or tissues and at which development stage the genes were highly expressed in standard conditions (Figures 3.9 and 3.10). With few exceptions, the selected genes displayed low expression on the diverse anatomic parts and developmental stages studied. As heat-stress specific genes, it would be expected that under standard conditions they will not be active or display a reduced expression.

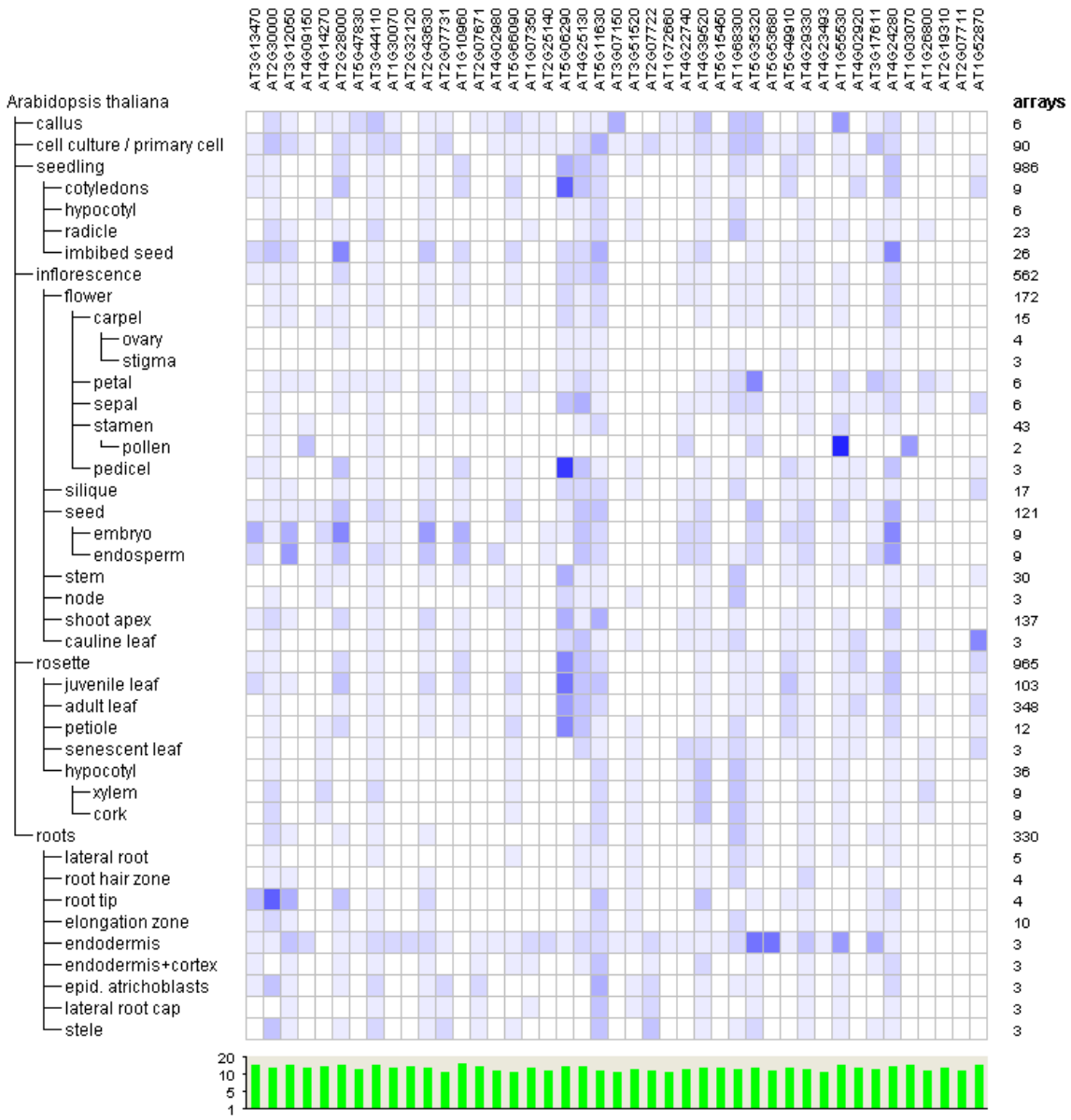


Figure 3.9. Expression profile of the *A. thaliana* 43 selected genes in different plant organs and tissues according to the Meta-profile analysis (Anatomy tool) of *Genevestigator*. Results are given as heat maps in blue/white coding (average expression levels), in which blue intensity indicates the expression level. The level of variance within each condition is indicated for each gene below the heat map and the number of arrays considered to calculate the mean value is displayed at right.

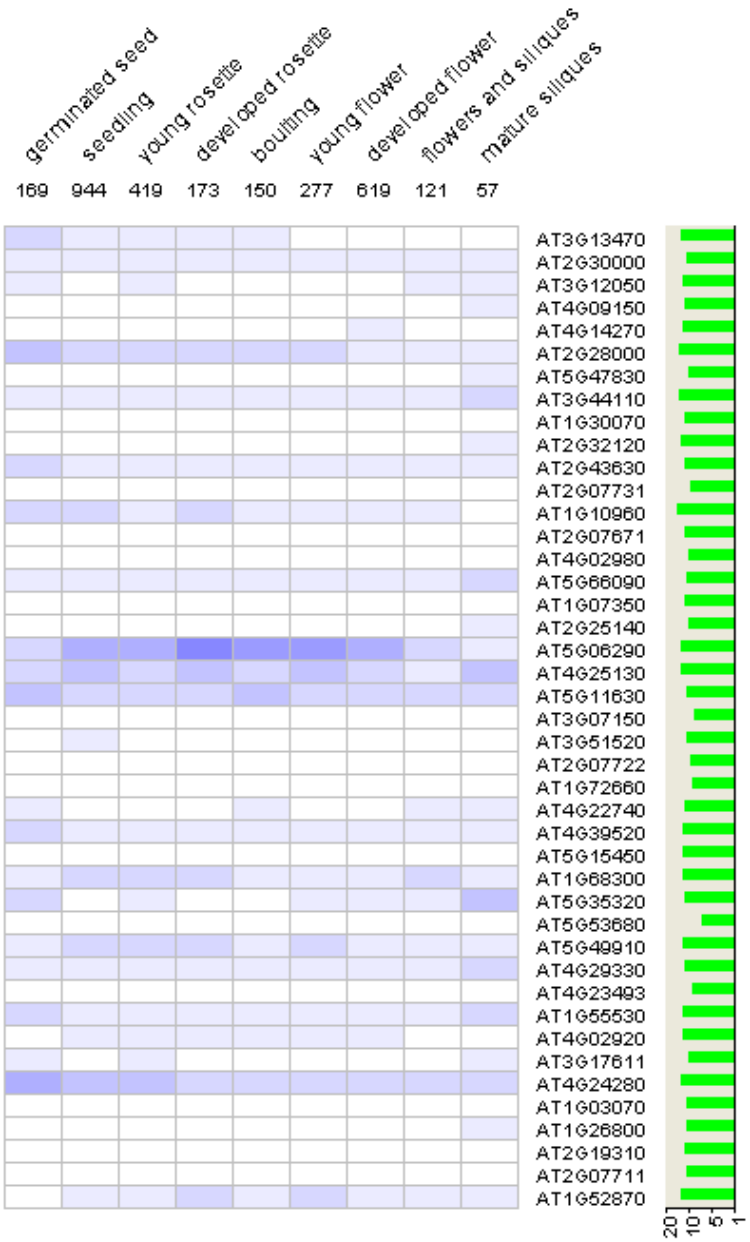


Figure 3.10. Expression profile of the *A. thaliana* 43 selected genes in different developmental stages according to the Meta-profile analysis (Development tool) of *Genevestigator*. Results are given as heat maps in blue/white coding (average expression levels), in which blue intensity indicates the expression level. The level of variance within each condition is indicated for each gene at right and the number of arrays considered to calculate the mean value is displayed above the heat map.

Selected determinants displayed diverse functional categories

The selected 43 heat-stress specific genes were analyzed according to their functional classification using *Gene Ontology* (Ashburner *et al.* 2000). The predicted assignments for each selected gene were provided by TAIR and are summarized in Table 3.3. In this resource, most gene annotations are made by biocurators using the

Table 3.3 Functional assignment of the 43 heat-specific selected genes, according to the information available in TAIR. Gene annotations were grouped according to the biological process in which genes could be involved, as determined by their GO functional annotation. The corresponding GO terms related to cellular component and molecular function are displayed, as well significant references on gene characterization.

AGI [annotation]	Cellular Component	Molecular Function	References
Stress Response			
At1g68300[1] [universal stress protein (USP) family protein]	-	-	Kerk *et al.* (2003)
Abiotic Stress Response			
At2g19310[2] [similar to HSP18.2 At5g59720.1 [*Arabidopsis thaliana*]; similar to 18.5 kDa class I HSP 18.5; contains InterPro domain HSP20-like chaperone (InterPro:IPR008978)]	-	-	Taki *et al.* (2005) Nishizawa *et al.* (2006) Luhua *et al.* (2008)
At2g25140 [CLPB4, belongs to the Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100) family]	Chloroplast Mitochondrion	ATP binding ATPase activity	Keeler *et al.* (2000) Sokolenko *et al.* (2002) Carr *et al.* (2006) Lee *et al.* (2006) Zybailov *et al.* (2008)
At5g06290 [2-cystein peroxiredoxin B]	Chloroplast Apoplast	Antioxidant activity Peroxiredoxin activity	Dietz *et al.* (2002) Horling *et al.* (2003) Dietz *et al.* (2006) Goulas *et al.* (2006) Zybailov *et al.* (2008)
and Protein Folding			
At2g32120 [HSP70T-2]	-	ATP binding	Sung *et al.* (2001) Nishizawa *et al.* (2006) Charng *et al.* (2007)
At4g24280 [CPHSC70-1, chloroplast heat shock protein 70-1]	Chloroplast Mitochondrion Nucleus	ATP binding	Sung *et al.* (2001) Jackson-Constan and Keegstra (2001) Bae *et al.* (2003) Zybailov *et al.* (2008) Su and Li (2008)
At5g49910 [CPHSC70-2, chloroplast heat shock protein 70-2]	Chloroplast	ATP binding Protein binding	Sung *et al.* (2001) Rios *et al.* (2002) Zybailov *et al.* (2008) Su and Li (2008)
and Chloroplast Organization			
At5g15450 [APG6, Albino and Pale Green 6]	Chloroplast	ATP binding ATPase activity	Keeler *et al.* (2000) Sokolenko *et al.* (2002) Lee *et al.* (2006) Myouga *et al.* (2006) Zybailov *et al.* (2008)
Proteolysis			
At4g29330[1] [DER1 (Derlin-1)]	-	-	-
Protein Modification Process			
At4g25130[1] [peptide methionine sulfoxide reductase, putative]	Chloroplast	Oxidoreductase activity	Peltier *et al.* (2002) Zybailov *et al.* (2008)
Protein Folding			
At3g13470[1] [chaperonin, putative]	Chloroplast Mitochondrion Nucleus Ribosome	ATP binding Protein binding	Kubis *et al.* (2003) Heazlewood *et al.* (2003) Zybailov *et al.* (2008)
At3g44110 [ATJ3 (*Arabidopsis thaliana* DnaJ homologue 3)]	Nucleus Membrane Cell wall	Protein binding	Zhou and Miernyk (1999) Zhou *et al.* (1999) Zhou *et al.* (2000) Hilson *et al.* (2004) Li *et al.* (2005)

Table 3.3 (continuation)

AGI [annotation]	Cellular Component	Molecular Function	References
and Chloroplast Organization/Embryogenesis			
At2g28000 [60 kDa chaperonin alpha involved in Rubisco folding]	Chloroplast Mitochondrion Ribosome Membrane	ATP binding Protein binding	Viitanen *et al.* (1995) Koumoto *et al.* (1996) Yang and Poovaiah (2000) Apuya *et al.* (2001) McElver *et al.* (2001) Millar *et al.* (2001) Heazlewood *et al.* (2003) Baud and Graham (2006) Zybailov *et al.* (2008)
RNA Splicing			
At1g07350[1] [transformer serine/arginine-rich ribonucleoprotein, putative]	-	RNA binding	Bosco *et al.* (2003) de la Fuente van Bentem *et al.* (2006)
Proton Transport			
At2g07671[1] [H^+-transporting two-sector ATPase, C subunit family protein]	Membrane	ATPase activity	Kamauchi *et al.* (2005)
Cell Growth and Cell Division			
At4g02980 [ABP1, auxin binding protein 1)]	Endoplasmic reticulum	Auxin binding	Shimomura *et al.* (1993) Anai *et al.* (1997) Jones *et al.* (1998) Chen *et al.* (2001) Steffens *et al.* (2001) Carrari *et al.* (2001) Chen *et al.* (2001) Braun *et al.* (2008)
Unknown			
At1g03070[1] [glutamate binding]	-	Glutamate binding	-
At1g10960 [ATFD1 (FERREDOXIN 1); 2 iron, 2 sulfur cluster binding/electron carrier/iron-sulfur cluster binding]	Chloroplast	Electron carrier activity	Vorst *et al.* (1990) Elo *et al.* (2003) Hanke *et al.* (2003) Hanke *et al.* (2005) Hanke and Hase (2008) Voss *et al.* (2008)
At1g26800[1] [zinc finger (C3HC4-type RING finger) family protein]	-	Ion binding Protein binding	Kosarev *et al.* (2002)
At1g30070[1] [SGS domain-containing protein]	Membrane	-	-
At1g52870[1] [peroxisomal membrane protein-related]	Chloroplast Membrane	-	-
At1g55530[1] [zinc finger (C3HC4-type RING finger) family protein]	-	Ion binding Protein binding	Menges *et al.* (2002)
At1g72660[2] [developmentally regulated GTP-binding protein, putative]	Intracellular	GTP binding	Rizhsky *et al.* (2004)
At2g07711[1] [pseudogene, similar to NADH dehydrogenase subunit 5]	-	-	-
At2g07722[1] [Identical to uncharacterized mitochondrial protein AtMg00170/AtMg00620 [*Arabidopsis thaliana*]; similar to hypothetical protein NitaMp112 [*Nicotiana tabacum*]]	-	-	-
At2g07731[1] [pseudogene, similar to NADH-ubiquinone oxidoreductase chain 6]	-	-	Cartieaux *et al.* (2003)

Table 3.3 (continuation)

AGI [annotation]	Cellular Component	Molecular Function	References
At2g30000[1] [Identical to uncharacterized protein At1g07170/At2g30000 [*Arabidopsis thaliana*]; similar to Os04g0663300 [*Oryza sativa* (japonica cultivar-group)]; similar to unknown [*Picea sitchensis*]; contains InterPro domain PHF5-like (InterPro:IPR005345)]	-	-	Hampton *et al.* (2004)
At2g43630[1] [similar to glycine-rich protein At3g59640.2 [*Arabidopsis thaliana*]]	Chloroplast Nucleus	-	Peltier *et al.* (2004) Zybailov *et al.* (2008)
At3g07150[1] [similar to hypothetical protein [*Oryza sativa* (japonica cultivar-group)]]	-	-	-
At3g12050[2] [Aha1 domain-containing protein]	Cytoplasm	ATPase activator activity Protein binding	Charng *et al.* (2007)
At3g17611[1] [rhomboid family protein/zinc finger protein-related]	Intracellular Membrane	Ion binding	Tripathi and Sowdhamini (2006) Kmiec-Wisniewska *et al.* (2008)
At3g51520[1] [diacylglycerol acyltransferase family]	-	Transferase activity	-
At4g02920[1] [similar to unknown protein At1g03340.1 [*Arabidopsis thaliana*]; similar to IMP dehydrogenase/GMP reductase [*Medicago truncatula*]]	-	-	Vlieghe *et al.* (2003)
At4g09150[1] [T-complex protein 11]	-	-	-
At4g14270[1] [Protein containing PAM2 motif which mediates interaction with the PABC domain of polyadenyl binding proteins]	-	-	Bravo *et al.* (2005) Wenzel *et al.* (2008)
At4g22740[1] [glycine-rich protein; similar to unknown protein At2g45380 [Arabidopsis thaliana]]	Membrane	-	Berg *et al.* (2003)
At4g23493[1] [unknown protein]	-	-	Lan *et al.* (2007)
At4g39520[1] [GTP-binding protein, putative]	Intracellular	GTP binding	Testerink *et al.* (2004)
At5g11680[1] [similar to hypothetical protein Os09g0512900 [*Oryza sativa* (japonica cultivar-group)]]	Cytoplasm Membrane	-	Borderies *et al.* (2003) Hameister *et al.* (2007)
At5g35320[1] [similar to conserved hypothetical protein [*Medicago truncatula*]]	-	-	-
At5g47830[1] [similar to hypothetical protein MtrDRAFT_AC150207g26v2 [*Medicago truncatula*]]	-	-	-
At5g53680[1] [RNA recognition motif (RRM)-containing protein]	-	RNA binding	-
At5g66090[1] [similar to Os09g0541700 [*Oryza sativa* (japonica cultivar-group)]; contains domain adenine nucleotide alpha hydrolases-like (SSF52402)]	Chloroplast	-	Bosco *et al.* (2003) Zybailov *et al.* (2008)

AGI: *A. thaliana* genome initiative number

[1] Genes without information on gene/protein function and not previously associated with heat stress responses.

[2] Genes without information on gene/protein function but previously associated with heat stress responses.

published literature as sources, whereas other annotations are based upon computational methods. Functional annotations are described using the specific GO terms, according to a) biological process, b) cellular component and c) molecular function. A gene product might be associated with or located in one or more cellular components; it could be active in one or more biological processes, during which it performs one or more molecular functions. Despite the majority of selected heat-stress specific genes do not have predicted functional information, a broad range of functional categories are still present (Figure 3.11). According to the biological process, many of the selected genes appear to be strongly implicated in stress response (eight genes), six of which have already been associated to heat stress responses (Figure 3.11A, Table 3.3). Additionally, other eight genes have a particular role in protein metabolism (mainly in protein folding), which is widely known to be an important adaptive mechanism that protects cells from the effects of heat stress (reviewed by Wahid *et al.* 2007). The increased production and accumulation of heat shock proteins along with other heat inducible proteins have been observed under elevated temperature in many studies. Although the mechanism of heat tolerance is still unclear, these proteins appear to enhance tolerance by insuring the proper folding of new and denatured proteins under heat stress allowing them to function properly. They could also be involved in transporting proteins through cell compartments or removal of undesired proteins through proteolysis. Concerning the subcellular localization, there are many genes that seem to be intracellular, mainly directed to chloroplast, or associated to membranes (Figure 3.11B). The other selected genes are

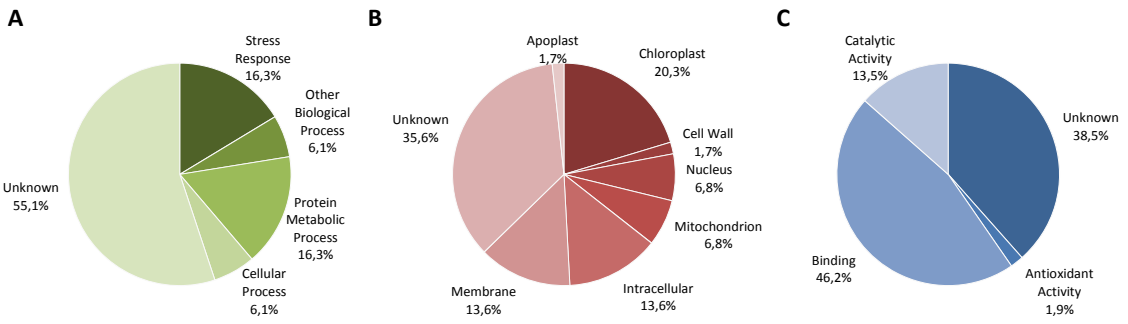


Figure 3.11. Functional classification of the 43 selected heat up-regulated specific genes, according to biological process (A), cellular component (B) and molecular function (C). Analysis was performed using the GO functional annotations provided by TAIR for each selected gene. Results are presented as percentage of category frequency.

included in diverse cellular component categories (nucleus, mitochondrion, apoplast and cell wall). However, the majority of the genes do not seem to be associated to a known biological process or a cellular component. According to the molecular function, although a great part of the selected genes does not have predictable information, the majority of the selected genes display binding capacity to a wide variety of molecules (nucleotide, protein, zinc ion, RNA, auxin, aminoacid and metal clusters) (Figure 3.11C). Additionally, a few number of genes present catalytic activity.

Two determinant genes were singled out from expression analysis

In order to determine the actual state-of-the-art of each selected heat-stress associated gene, a literature search in TAIR was performed (Table 3.3). The preference for performing further studies on functional characterization was given to the genes with no available information on gene/protein function and whose implication in heat stress response has not been investigated yet (30 genes, Table 3.3). From these, two genes were selected: *HZF* (At1g26800) that encodes a putative zinc finger family protein (C3HC4-type RING finger) and *HRR* (At5g53680) that encodes a putative protein containing a RNA recognition motif (RRM). *HZF* gene product was only referred in a single study concerning analysis of the *Arabidopsis* proteome, in which 387 proteins containing RING-finger domains were identified (Kosarev *et al.* 2002). In this study, cluster analysis of the RING domains according to their metal-ligand arrangement defined groups of proteins, which frequently show significant similarity outside the RING domain. A common evolutionary origin and potential overlapping functionality of group members was then suggested. For the *HRR* gene no significant relevant publication was found.

Using the previously accessed microarray data (*heat stress time course* experiment, included in the *AtGenExpress abiotic stress series*) the expression response profile of each gene was determined (Figure 3.12). For both genes, an induction of gene expression was observed just after heat stress imposition. This was observed in all biological samples (shoots, roots and cell suspensions). The expression profile of both genes in cell suspension was very similar. The maximal expression levels were detected one hour after heat stress imposition and gradually decreased to basal

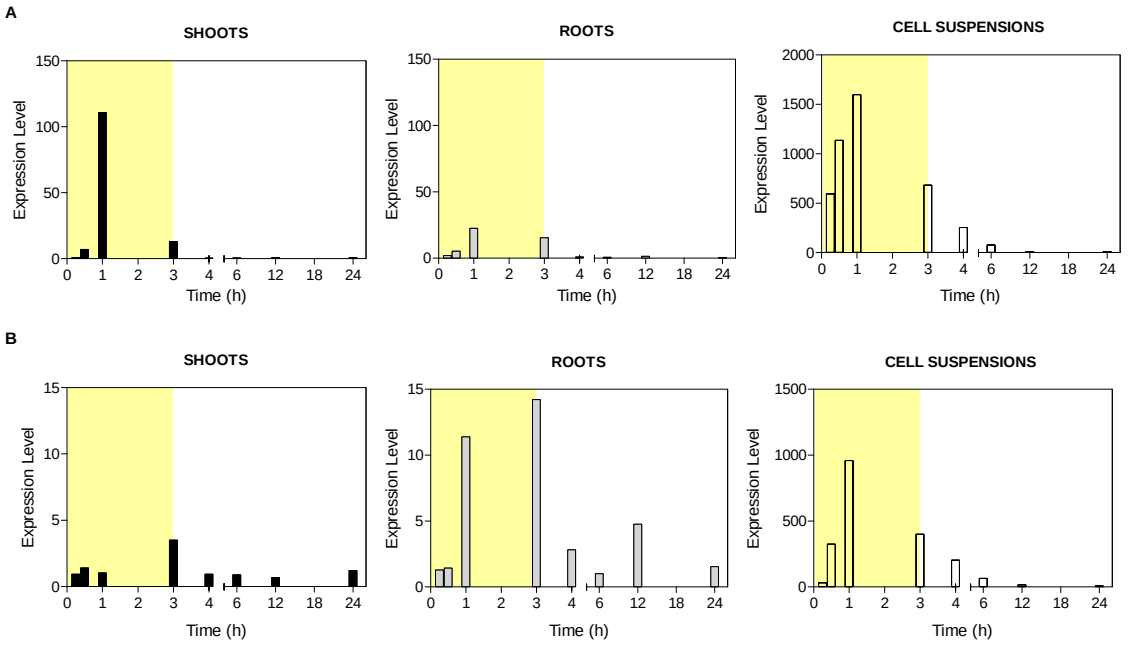


Figure 3.12. Expression response profiles of *HZF* (A) and *HRR* (B) genes on *A. thaliana* shoots, roots and cell suspensions after a heat stress (38°C) treatment. The data was obtained from the *heat stress time course* experiment, included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007). After 3 h of light treatment, 16-day-old plants or 6-day-old cell suspensions were subjected to a heat stress of 38°C for 3 h (highlighted in yellow) and were then allowed to recover at 25°C. Expression levels are displayed as relative values (pixel count in heat stressed cells/pixel count in control cells).

values during recovery at 25°C. A different response profile was found for each gene in shoots and roots samples.

The *HZF* gene presented a typical early response profile, both in shoots and roots, reaching its maximal expression level 1 h after heat stress imposition and decreasing afterwards (Figure 3.12A). This profile was mainly evident in shoots, suggesting a functional role connected to the aerial part of the plant. Although presenting higher expression values during the first hour of heat treatment, *HZF* gene must have been included in class IV responsive genes (Figure 3.4), since it still displays high expression levels after 3 h of heat stress.

The *HRR* gene also displayed an increase of the expression level in both shoots and roots, which started just after heat stress imposition and continued to increase up to 3 h (Figure 3.12B). During recovery at 25°C, *HRR* expression still continued. A slightly different response was detected between both biological samples. In shoots, a more than two-fold significant increase in gene expression was only detected 3 h after treatment (a typical class II responsive gene, Figure 3.4). In roots, significant gene up-

regulation started earlier, attained maximal levels 3 h after treatment and maintained significant expression levels until the end of the treatment (a typical class VII responsive gene, Figure 3.4). In opposition to *HZF* gene, the induction of *HRR* gene was much stronger in roots, where it can play a functional role.

Previously described databases and tools were explored in order to assemble information of *HZF* and *HRR* genes. Gene structure and other relevant gene features were either obtained from TAIR or MAtDB databases (Table 3.4). A detailed list of the most co-regulated genes for each gene, provided by ATTED-II resource, is depicted in Table 3.5. *HZF* gene expression appears to be strongly correlated with the expression of genes implicated in transcriptional regulation. Among the ten genes presenting the highest correlation values, the majority encode transcription factors (four genes). Additionally, one putative kinase and one putative heat shock protein are also co-regulated with *HZF* gene. As already mentioned, the initiation of heat stress response depends on the action of transcription factors, protein kinases and phosphoinositide metabolism enzymes (reviewed by Kaur and Gupta 2005). The results provided by ATTED-II database thus suggest the implication of *HZF* gene in the initial phase of heat response, which is in agreement with the previous findings on gene expression

Table 3.4 Main gene attributes available on bioinformatics resources (TAIR and MAtDB) for the two selected potential thermotolerance determinant genes.

	HZF	***HRR***
Chromosome number	1	5
Strand	Reverse	Forward
Start position	9285086	21815608
End position	9286311	21816336
Sequence length (bp)	1226	728
Number of exons	1	3
Annotation		
Biological process	Unknown	Unknown
Cellular component	Unknown	Unknown
Molecular function	Zinc ion binding Protein binding	RNA binding Nucleic acid binding
Protein		
Length (aa)	204	169
Molecular weight	22735.1	19588.9
Domains	Zinc finger, RING/FYVE/PHD-type Zinc finger, RING-type	Nucleotide binding, alpha-beta plait RNA recognition motif, RNP-1

Table 3.5 Top ten co-expressed genes of selected potential heat determinant genes, as predicted by ATTED-II resource, ranked according to their correlation values. Those genes highlighted in yellow were previously found in commonly up-regulated 137 genes, while the only gene highlighted in grey was previously selected as one of the 43 most heat-specific genes (Table 3.2).

	Co-expressed gene	Function
HZF	At3g51910	Heat shock transcription factor A7A
	At1g14200	Zinc finger (C3HC4-type RING finger) family protein
	At3g10020	Unknown protein
	At2g26150	Heat shock transcription factor A2
	At3g24500	Multiprotein bridging factor 1C
	At2g21940	Shikimate kinase, putative
	At1g55530	Zinc finger (C3HC4-type RING finger) family protein
	At5g37710	Lipase class 3 family protein/calmodulin-binding HSP, putative
	At4g11660	Heat shock transcription factor B2B
	At4g05070	Unknown protein
HRR	AtMg00600	Unknown protein (mitochondrial)
	At2g44170	N-myristoyltransferase 2, pseudogene
	At4g25380	Zinc finger (AN1-like) family protein
	At5g15250	FtsH protease 6
	At2g13890	Transposable element gene
	At2g04670	Transposable element gene
	At3g63350	Heat shock transcription factor A7B
	At1g33500	Unknown protein
	At3g56250	Unknown protein
	At5g18340	U-box domain-containing protein

analysis. Also, two similar zinc finger (C3HC4-type RING finger) family proteins display high correlation values. Concerning *HRR* co-expressed genes, a variety of different transcripts appear to have their expression correlated with this gene. It is important to notice that both genes, but mostly *HZF*, showed co-expression with various heat shock transcription factors, already established to have essential roles in the regulation of gene expression under heat stress (reviewed by Von Koskull-Döring *et al.* 2007). This fact reveals the efficiency of the strategy in identifying genes determinant for heat responses.

Additional gene information, as promoter sequences and putative *cis*-acting elements, can be of assistance to elucidate associated transcriptional regulatory networks and was predicted in AtcisDB provided by AGRIS resource. For *HZF* and *HRR* genes, predicted promoter regions and annotation information were retrieved in two different ways by using this tool: a graphical organization of the promoter regulatory regions, showing the predicted transcription factor binding sites represented by a set

of small grey squares (Figure 3.13) and a table list with the description of each predicted binding site with their localization in the genome, sequence and presumed associated transcription factor family (Table 3.6). The predicted regulatory elements of both genes are located upstream from the transcription start site (Figure 3.13). The presence of heat shock elements (HSE) that are usually found in the promoters of

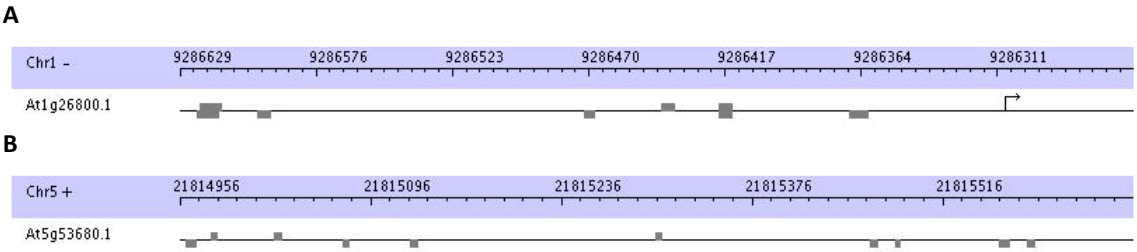


Figure 3.13. Graphic representation of the regulatory regions of *HZF* (A) and *HRR* (B) promoter. An overview of gene localization in the genome is highlighted in blue. Predicted *cis*-acting elements are represented by grey boxes and the position of the transcription start site is indicated by a small arrow. Detailed information of gene binding sites is described in Table 3.6. Image was acquired using the AtcisDB displayed by AGRIS resource.

Table 3.6 Binding sites (BS) present on the promoters of the selected potential heat determinant genes (*HZF* and *HRR*), as predicted by AtcisDB provided by AGRIS.

	BS name	BS genome start	BS sequence	Associated TF family
HZF	ATHB5 binding site motif	9286614	caatcattg	HB
	ATHB5 binding site motif	9286613	caatgattg	HB
	RAV1-A binding site motif	9286468	caaca	AP2 (RAV1)[1]
	LFY consensus binding site motif	9286415	ccagtg	LFY
	LFY consensus binding site motif	9286415	ccactg	LFY
	ABRE-like binding site motif	9286362	tacgtgta	-
	Ibox promoter motif	9286594	gataag	-
	Ibox promoter motif	9286437	gataag	-
HRR	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-A binding site motif	21814979	caaca	AP2 (RAV1)[1]
	RAV1-A binding site motif	21815481	caaca	AP2 (RAV1)[1]
	RAV1-A binding site motif	21815076	caaca	AP2 (RAV1)[1]
	BoxII promoter motif	21815578	ggttaa	-
	GATA promoter motif [LRE]	21815025	agataa	-
	GATA promoter motif [LRE]	21815463	agataa	-
	Ibox promoter motif	21815026	gataag	-

[1] According to Kagaya *et al.* (1999), RAV1 transcription factors bind simultaneously to two unrelated binding sites motifs: RAV1-A (recognized by an N-terminal AP2 domain) and RAV1-B (recognized by a C-terminal ABI3/VP1 domain)
TF: transcription factor

heat-specific genes (Schöffl *et al.* 1998), in particular in heat shock proteins, was not detected.

According to Rombauts *et al.* (2003), genes that are similarly expressed in response to a given, unique stress might be coordinately regulated by common *cis*-elements. By analysing the predicted regulatory regions of *HZF* and *HRR* genes, only RAV1-A and Ibox motifs were commonly found as putative regulatory elements (Table 3.6). RAV1-A motif (caaca) has been documented as a potential binding site for RAV1 (from *related to ABI3/VP1*) transcription factor, which presents two distinct DNA-binding domains (AP2 and ABI3/VP1) (Kagaya *et al.* 1999). Although RAV1 transcription factor has been suggested to act as negative regulator of growth and development (Hu *et al.* 2004), no relation to heat stress has been established yet. The Ibox promoter motif is a six-bp sequence (gataag) present in the promoter of many light-regulated genes (Giuliano *et al.* 1988). Also, no involvement in the regulation of heat response has been reported yet for this binding site.

Another valuable tool for functional genomics studies is provided by BAR, in which the subcellular protein product localization can be predicted by creating electronic fluorescent pictographic representations of our genes of interest. The *HZF* gene product is strongly predicted to be localized in the cell nucleus (Figure 3.14A). *HRR* protein is also localized in the nucleus (Figure 3.14B), although in this particular case with a lower confidence score. For this gene, a minimum confidence scored prediction is also achieved for cytosol.

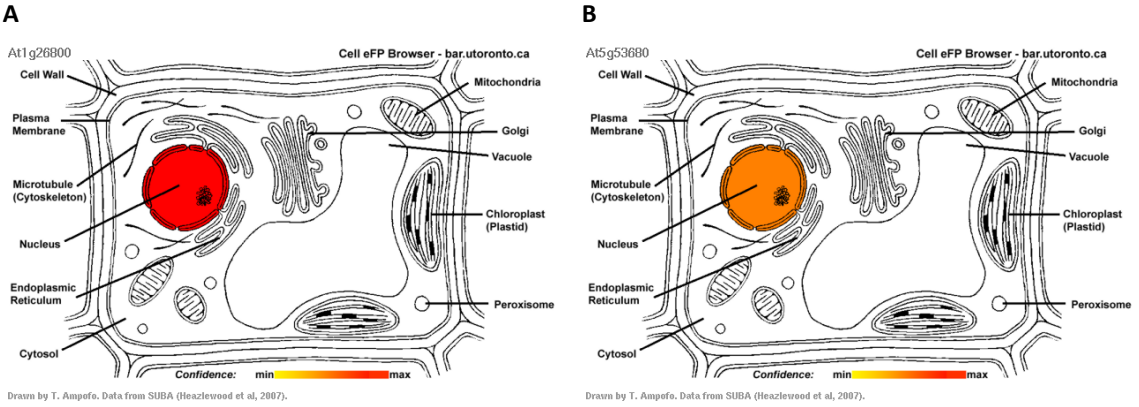


Figure 3.14. Prediction of subcellular localization of *HZF* (A) and *HRR* (B) gene products as an "electronic fluorescent pictographic" representation. This analysis was performed using Cell eFP Browser provided by BAR.

CONCLUSIONS AND FUTURE PERSPECTIVES

The broad collection of recently developed *Arabidopsis* resources and tools that can be easily accessed using the internet offers a powerful wealth of data, which can ultimately lead to the understanding of specific plant processes. Our proposed strategy was firstly based on the identification of differentially expressed genes under heat stressing conditions by using publicly available microarray data. The efficiency of AtGenExpress datasets for the identification of heat shock proteins and transcription factors specifically involved in the heat response pathway has been recently reported (Swindell *et al.* 2007). A significant number of other differentially expressed transcripts detected after heat treatment provided also helpful information to understand heat stress responses (reviewed by Huang and Xu 2008). In this work, microarray analysis led to the identification of a great number of heat stress up-regulated transcripts. For the selection of the most heat specific determinant genes, several tools were further used concerning expression pattern analysis and other information, leading to the final selection of two genes putatively involved in thermotolerance (*HZF* and *HRR*). According to the present knowledge of the heat shock response (reviewed by Kaur and Gupta 2005), and regarding the gene information available, both genes could encode effector proteins involved in re-establishing the cellular homeostasis or in the protection of proteins and membranes against heat stress, although in different periods of the process. Relevant information for the potential heat determinant genes *HZF* and *HRR* was collected, and would be subsequently used for the comprehension of their gene function and association with the heat stress response in *Arabidopsis* (Chapter 5).

The co-expressed genes network of *HZF* and *HRR* provided the first sign of the effectiveness of the strategy, since both genes were connected with genes already implicated in the regulation of heat responses (*e.g.*, HSFA2) (Schramm *et al.* 2006). A conclusive evidence of the success of this strategy will be accomplished after establishing a heat-associated phenotype response in loss-of-function mutants on *HZF* gene and further characterization of these genes (Chapter 5). The used outlined strategy, supported by a simple *in silico* approach, can be followed to speed up the

detection of other genes essential, not only for plant responses to heat, but also to other forms of stress.

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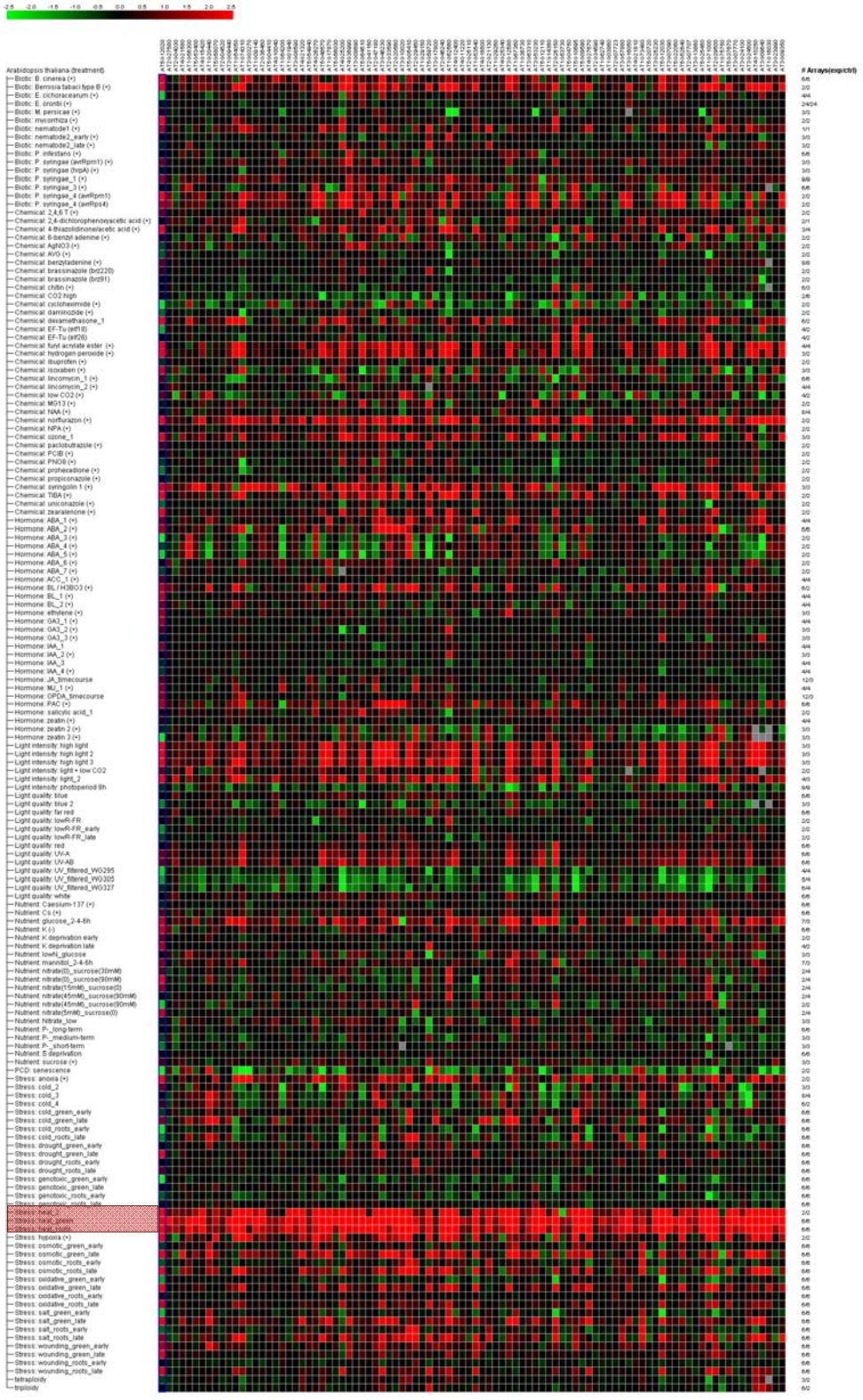
ANNEX I

Expression profile of those *A. thaliana* selected genes that display less heat specificity in response to different external biotic or abiotic stimuli, according to the Meta-profile analysis (Stimulus tool) of *Genevestigator*. Results are given as heat maps in red/green coding. Red indicates that the signal intensity of the treatment is higher than signal intensity of the corresponding control, and green means the opposite. AGI number is displayed on the top. Treatments are indicated on the left, where heat treatments are highlighted in red.



Chapter 4

Phenotypic analysis of *Arabidopsis* mutants: heat response assays

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CONTENTS

Phenotypic analysis of *Arabidopsis* mutants: heat response assays

ABSTRACT

Phenotypic characterization of *Arabidopsis thaliana* knockout mutants can be an arduous and unpredictable task which often involves the analysis of multiple phenotypic parameters. In particular, *Arabidopsis* thermotolerance has been evaluated by several groups based on direct assessments like germination rate, seedling survival, hypocotyl and root elongation or indirect measurements as chlorophyll and TBARS accumulation. A comprehensive examination of the literature reveals that the evaluation of heat effects on germination and seedling survival are the most common. In an attempt to simplify the detection of heat-associated phenotypes, a valuable collection of protocols to be used in a primary approach is proposed. Temperatures and lengths of treatment were combined to develop several thermotolerance assays to be used at seed and 7-day-old seedlings. Characterization of basal and acquired thermotolerance in wild-type Col-0 and Ws plants was initially performed to predetermine the finest assay conditions. Using the outlined protocols, the usefulness of this methodology was illustrated through the detection of heat-associated phenotypes in two mutants (*hot1-3* and *atrbohD*) previously identified to be thermotolerance defective.

KEYWORDS Acclimation · Acquired thermotolerance · *Arabidopsis thaliana* · Basal thermotolerance · Germination · Phenotype · Seedling survival

ABBREVIATIONS *atrbohD*: ***A. t****haliana* **r**espiratory **b**urst **o**xidase **h**omolog **D** mutant · Col-0: *A. thaliana* **Col**umbia ecotype · DNA: **d**eoxyribo**n**ucleic **a**cid · HSFA2: **h**eat **s**hock transcription **f**actor **A2** · HSP: **h**eat **s**hock **p**rotein · LST_{50}: temperature at which there is **50**% of surviving seedlings (**l**ethal **s**urvival **t**emperature) · LSt_{50}: treatment period at which there is **50**% of surviving seedlings (**l**ethal **s**urvival **t**ime) · LT_{50}: temperature at which there is **50**% of germinated seeds (**l**ethal **t**emperature) · Lt_{50}: treatment period at which there is **50**% of germinated seeds (**l**ethal **t**ime) · MES: 2-(N-**m**orpholino)-**e**thane **s**ulphonic acid · MS: **M**urashige and **S**koog · NADPH: **n**icotinamide **a**denine **d**inucleotide **p**hosphate · NASC: ***N****ottingham* ***A****rabidopsis* ***S****tock* ***C****entre* · ROS: **r**eactive **o**xygen **s**pecies · SEM: **s**tandard **e**rror of the **m**ean · TBARS: **t**hio**b**arbituric **a**cid **r**eactive **s**ubstances · T-DNA: **t**ransferred **DNA** · Ws: *A. thaliana* **W**assilew**s**kija ecotype

INTRODUCTION

Facing up to the new challenge of large-scale discovery of gene function in *Arabidopsis thaliana*, plant biology community has been presently focused on the improvement of reverse genetics methodologies and tools. One of the most critical steps in a reverse approach is to determine if a given mutation results in a phenotype distinct from the wild-type, which could give new insights on the function of that gene (Feng and Mundy 2006). Searching for a noticeable phenotype can be an arduous process often hindered by gene redundancy or subtle imperceptible changes in growth or development (Bouché and Bouchez 2001). The high-throughput determination of gene function led Boyes *et al.* (2001) to develop a sensitive and dynamic method to detect and interpret phenotypic alterations over the entire development of *Arabidopsis* plants. Based on defined stages of development and their associated traits, two complementary platforms were created: one directed for analysis of seedling growth on vertical plates and the other for characterization of plant growth on soil. Although highly helpful for phenotypic analysis of mutations that led to altered timing of development, this extensive temporal analysis could be circumvented if significant morphological changes can be easily identified. However, the detection of morphological differences is often clearly dependent on the application of a particular experimental condition. Mutations in certain genes may only display a detectable phenotype when subjected to specific environmental challenges or a combination of several conditions (Bouché and Bouchez 2001). Thus, the selection of the most suitable assay conditions could be critical for the success in the phenotype identification.

In the particular case of thermotolerance, a standardized experimental protocol designed specifically to detect temperature-related phenotypes has not been reported yet. As a consequence, distinct assays and conditions have been used by different laboratories (Table 4.1). The use of such different *in vitro* conditions could alter the severity of the stress and consequently have implications in the detection of the phenotype or in the comparison between different mutant phenotypes. Such conditions could be the intensity and duration of stress imposition, the growth stage of treated plants and the presence/absence of a pre-conditioning treatment.

Table 4.1 A non-exhaustive survey of the most commonly employed thermotolerance assays for phenotypic studies in thermosensitive *Arabidopsis* mutants.

Mutant	Thermotolerance tested	Growth stage	Assayed feature(s)	Reference(s)
***dgd1-2* (AtTS02)**	Basal/Acquired	Seedling	Chlorophyll accumulation	Burke *et al.* (2000) Chen *et al.* (2006*a*)
			Survival	
atts244	Basal	Seed	Germination	Chen *et al.* (2006*b*)
		Seedling	Cotyledon greening	
	Acquired	Seedling	Hypocotyl elongation	
			Survival	
			Chlorophyll accumulation	
hit1	Basal	Seed	Germination	Wu *et al.* (2000)
		Seedling	Maturation	
			Survival	
hot1	Basal	Seed	Hypocotyl elongation	Hong and Vierling (2000; 2001) Hong *et al.* (2003)
		Seedling	Hypocotyl elongation	
			Ion leakage	
	Acquired	Seedling	Hypocotyl elongation	
			Reporter enzyme activity	
			Ion leakage	
hsa32-1 ***hsfA2-1***	Basal/Acquired	Seedling	Survival	Charng *et al.* (2006; 2007)
			Ion leakage	
			Hypocotyl elongation	
***tfl2-6* (TU8)**	Basal/Acquired	Mature plant	Shoot elongation	Ludwig-Müller *et al.* (2000) Kim *et al.* (2004)
			Survival	
			Fresh weight	
Multiple heat-associated mutants	Basal	Seed	Germination	Larkindale *et al.* (2005*a*)
		Seedling	Survival	
			TBARS accumulation	
	Acquired	Seedling	Hypocotyl elongation	
			Root growth	
			Survival	
			TBARS accumulation	
		Mature plant	Survival	

atts244: ***A****rabidopsis* ***t****haliana* **t**hermo-**s**ensitive **244** mutant; *dgd1*: **dig**alactosyl**d**iacylglycerol synthase **1** mutant; *hit1*: **h**eat-**i**n**t**olerant mutant; *hot*: sensitive to **hot** temperatures mutant; *hsa32*: **h**eat-**s**tress-**a**ssociated **32**-kD protein mutant; *hsfA2*: **h**eat **s**hock transcription **f**actor **A2** mutant; *tfl2*: **t**erminal **fl**ower **2** mutant

A simple basic query a researcher may face when setting up a phenotypic analysis experiment is the temperature that should be used. This is often dependent on the length of treatment, since longer heat stresses at lower temperatures can produce similar effects as short treatments under higher temperatures. An examination over the published phenotypic studies associated with thermotolerance reveals that a stress treatment close to 45°C is preferentially used by researchers for detection of fitness alterations (Hong and Vierling 2000; Hong and Vierling 2001; Hong

et al. 2003; Larkindale *et al.* 2005*a;* Charng *et al.* 2006; Chen *et al.* 2006*b;* Charng *et al.* 2007; Larkindale and Vierling 2008). In these reports, the duration of the heat treatment was highly variable under the range of 15 to 220 min. However, the reason why this temperature is the most commonly applied is not completely understood, since no exhaustive assessment of thermotolerance in *Arabidopsis* wild-type plants was performed until now.

The type of thermotolerance involved (basal or acquired) and the way of shifting temperature (instantly or gradually) must be also considered for the experimental design, in order to enclose all the potential conditions that could generate a visible phenotype. Plants can rapidly acquire thermotolerance to otherwise lethal extreme temperatures, if they are pre-exposed to a moderate high temperature or exposed to a gradual increase in temperature. This phenomenon known as acquired thermotolerance is clearly distinct from basal thermotolerance, which refers to the innate ability of plants to survive exposure to temperatures above the optimal for growth (Larkindale *et al.* 2005*b*). Using *in vitro* assays, the acclimation treatment is usually performed at a moderate temperature around 38°C for 60-90 min (Hong and Vierling 2000; Hong and Vierling 2001; Hong *et al.* 2003; Larkindale *et al.* 2005*a;* Charng *et al.* 2006; Chen *et al.* 2006*b;* Charng *et al.* 2007). Currently, there are a number of reports that perfectly illustrates the effect of combining diverse conditions to evaluate the individual response of basal and acquired thermotolerance (Larkindale *et al.* 2005*a;* Charng *et al.* 2006; Chen *et al.* 2006*b;* Charng *et al.* 2007; Larkindale and Vierling 2008). Temperature and duration of the stress imposition, application of a pre-conditioning treatment (which can differ in temperature and/or in time length) and the duration of the recovery period are only some of the conditions that could be arranged in multiple ways to establish the phenotypic assay. For example, in a recent study performed by Larkindale and Vierling (2008), plants subjected to a severe heat stress treatment, either without acclimation or using two distinct acclimation treatments (by a gradual or a step-wise increasing in temperature) displayed different degrees of seedling survival. As demonstrated by Charng *et al.* (2007), the duration of the recovery period between the pre-conditioning treatment and the severe heat stress can hamper the phenotype detection as well. Accordingly, the *HSFA2* gene mutant line

displayed less tolerance to severe heat challenge than the wild-type after using extended recovery periods (Charng *et al.* 2007).

Along with the many interacting factors that can be introduced into the experimental design, the detection of phenotypic alterations can be assessed using different parameters. The most frequent are the evaluation of seedling survival, germination rate and hypocotyl/root elongation. But others, like accumulation of chlorophyll or TBARS, assessment of membrane permeability by ion leakage and activity of a reporter enzyme are also found in the literature (Table 4.1). Another aspect to be considered, which is frequently underestimated, is the time point for determining plant traits. Even for similar morphological assessments, some researchers obtain the results a few days after heat treatment, while others wait for several days. Therefore, it is significantly important to perform a time-course analysis of the phenotypic traits in question, which can reveal a phenotype *per se*, to facilitate the discovery of masked alterations between mutant and wild-type plants.

The growth stage of the treated plant also interferes with the recognition of phenotypic alterations. While the analysis performed in seeds is regularly restricted to germination rate evaluation, different assessments could be achieved both in early (2.5 to 10-day-old) or late (10 to 25-day-old) seedlings. Larkindale *et al.* (2005*a*) tested plants at several stages of development to study basal and acquired thermotolerance of 45 *Arabidopsis* mutants. Basal thermotolerance was examined in seeds and 7-day-old seedlings by germination and seedling survival assays, respectively. Early seedlings were used for acquired thermotolerance tests in hypocotyl and root assays (2.5 and 4-day-old seedlings, respectively), whereas 7 and 25-day-old seedlings were used for evaluating viability. The extent of oxidative damage was also assessed in 7-day-old plantlets, without or following an acclimation treatment, as a measure of heat sensitivity. Whenever possible, the expression data of the considered gene must be previously analysed at different periods of plant development, to reduce the range of possible testing conditions.

The diversity of protocols outlined here provides a supportive basis to perform a reasonable search for heat-associated phenotypes. However, the use of such a high number of different protocols is extremely time-consuming, besides being often unproductive. In order to create a simplified framework procedure for the phenotypic

detection of heat-associated mutants, it is necessary to assess firstly the thermotolerance features in *Arabidopsis* wild-type plants. Motivated by the same purpose, Burke *et al.* (2000) characterized the acquired thermotolerance in Col-0 seedlings by chlorophyll accumulation assays. However, to our knowledge there is no study that characterizes the temperature response of *Arabidopsis* based on the assessment of germination and seedling survival traits. At which temperatures seeds fail to germinate, what are the temperatures that cause stress in seedlings, what is the best pre-conditioning treatment (temperature, time length and recovery period) for revealing the acquired thermotolerance, are some of the questions that had to be answered prior to the phenotypic analysis. It was our aim to develop a protocol for the characterization of the temperature responses in wild-type seeds and seedlings. The analysis of *Arabidopsis* basal responses was performed varying the temperature and length of treatment and evaluated by germination and seedling survival. Acquired thermotolerance was also characterized by analysis of seedling survival after different acclimation temperatures. The protocols described here provide a comprehensive primary approach for identifying and interpreting phenotypic differences of heat-associated mutants. The effectiveness of these assays was confirmed by phenotype detection in *hot1-3* (Hong and Vierling 2001) and *atrbohD* (Torres *et al.* 2002) mutant plants that had previously been found to have temperature-associated phenotypes.

MATERIAL AND METHODS

Plant material

Wild-type seeds of *Arabidopsis thaliana* ecotypes Columbia (Col-0) and Wassilewskija (Ws) were ordered from the public stock center NASC (http://arabidopsis.info/; Scholl *et al.* 2000). For testing the thermotolerance assays, seeds from two distinct insertion T-DNA lines were used. The homozygous mutant seeds of *hot1-3* (Hong and Vierling 2001) and *atrbohD* (Torres *et al.* 2002) were gently provided by E. Vierling (Department of Biochemistry & Molecular Biophysics, University of Arizona, USA) and M. A. Torres (Department of Biology, University of North Carolina, USA), respectively. Both insertion mutant lines were established in Col-0 background.

Seed sterilization and germination in sterile conditions

A. thaliana seeds of each previously mentioned line were stratified by immersion in water and incubation at 4°C for 3 days. Surface-sterilization was initiated by soaking seeds in 1 ml of 70% (v/v) ethanol for 5 min. Ethanol was replaced by 1 ml of bleach solution [20% (v/v) commercial bleach with 3.5% (w/v) effective chloride; 0.1% (v/v) Tween-20] and seeds were further incubated for 10 min. Occasional vortexing was performed during incubations. Subsequent washes with 1 ml of sterile distilled water were performed for 5 times to rinse out the bleach solution. For appropriate discarding of solutions, seeds were retained in the bottom of the tube by centrifugation at 10,000 *g* for 1 min (Centrifuge 5415C, Eppendorf). Finally, seeds were resuspended in a sterile 0.08% (w/v) agarose solution and sprinkled one-by-one in 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 and 0.8% (w/v) agar at pH 5.7. The plates were sealed with parafilm to prevent desiccation and placed vertically (except for thermotolerance assays) into the growth room (16 h light/8 h dark long photoperiod; 40 $\mu E.m^{-2}.s^{-1}$ light intensity; 23°C). All procedures were performed under sterile conditions in a horizontal laminar flow chamber (OSN).

Planting on soil for bulk seed production

Ten-day-old seedlings of wild-type (Col-0 and Ws) and mutant (*hot1* and *atrbohD*) lines, germinated at the same time, were transferred into a soaked 4:1 mixture of soil (Siro) and vermiculite (Asfaltex) to achieve synchronized seed production for effective phenotypic assays. The pots were covered with plastic wrap to keep humidity and to allow plantlets acclimation and were maintained in the growth room (16 h light/8 h dark long photoperiod; 40 $\mu E.m^{-2}.s^{-1}$ light intensity; 23°C). After 2 days, the plastic wrap was removed and plantlets were watered every 2 days until approximately 6 weeks of growth. When the siliques appeared completely dehydrated (~8 weeks of growth), the seeds were harvested using a thin metallic sieve for better separation from the other plant tissues. Collected seeds were maintained in the dark, at room temperature, in firmly sealed individual containers to prevent rehydration.

Thermotolerance assays – evaluating germination

The seed thermotolerance assays were performed following stratification and sterilization as described above. Previously sterilized seeds of wild-type and mutant lines were heat-stressed, immediately after removal from 4°C, by immersion of the corresponding microtubes into a temperature-controlled water bath. Two different assays were performed: a) by altering the extent of heat stress treatment (15-300 min) in a constant temperature of 50°C, and b) by incubating at different temperatures (38-56°C) for 60 min. Subsequently, the seeds were plated and allowed to germinate as referred above. After 2-3 days, the number of seeds showing radicle emergence was counted every day over a total period of 9-11 days. Photographs were taken at the end of the assay. The percentage of germination was determined as a function of untreated seeds germinated from the same wild-type/mutant line. Mean and SEM were derived from four independent replicates for each genotype tested, all containing 30 seeds from each heat stress treatment applied.

Thermotolerance assays – evaluating seedling survival

Seven-day-old seedlings of wild-type and mutant lines were obtained as described above. Heat treatments were performed by submersion of the sealed plates into a temperature-controlled water bath. Distinct assays were performed: a) by

altering the extent of heat stress treatment (5-45 min) in a fixed temperature of 45°C, b) by incubating at different temperatures (40-50°C) for 20 min and c) by pre-incubating the seedlings at different temperatures (30-40°C) for 60 min with subsequent recovery at 23°C for 120 min, and then heat-treating the seedlings by plate submersion at 45°C for 20 min. At the end of all thermotolerance assays, the seedlings were returned to the growth room immediately after treatment and allowed to recover for 6 days. The plates were then photographed and the number of viable seedlings counted for determination of survival rate. Seedlings that were still green and generated new leaves were scored as surviving. Mean and SEM were derived from three independent replicates for each heat stress treatment applied, all containing 100 seedlings of each genotype tested.

CHARACTERIZATION OF THERMOTOLERANCE IN *ARABIDOPSIS* WILD-TYPE PLANTS

The assessment of basal thermotolerance was performed in wild-type *A. thaliana* seeds (Col-0 and Ws ecotypes) by evaluating their germination capacity. Basal and acquired thermotolerance were evaluated using 7-day-old seedlings of the same ecotypes by determining their survival ability upon heat stress imposition.

Basal thermotolerance evaluation by germination assays

In vitro seed germination is dependent on well-defined assay conditions, like the growth medium, availability of water and oxygen, light quality and temperature conditions (Bove *et al.* 2001). Maintaining the incubation medium, water, oxygen and light intensity controlled, the heat sensitivity of wild-type seeds (Col-0 and Ws) was evaluated by their germination rate, upon treatment at 50°C during different periods (15, 30, 60, 120, 180, 240 and 300 min) (Figure 4.1). Since the germination process is defined as the events occurring after imbibition until visible protrusion of the radicle (Nonogaki *et al.* 2007), a germinated seed was considered when the emergence of the radicle was observable. The time-course evaluation of the number of germinated seeds allowed to notice a germination delay caused by longer periods of heat stress (Figure 4.1A). The maximal germination in the untreated seeds was nearly achieved 2-4 days after heat stress, whereas the heat-stressed seeds required distinct periods to accomplish it, which were dependent on the duration of treatment. Therefore, for detecting germination alterations upon heat treatment care should be taken on the assessment moment.

Considering the data obtained after 10 days of heat treatment and seed plating, a significant germination decline was considered to occur for those heat treatments below Lt_{50} (lethal time), corresponding to the treatment period at which there is 50% of germinated seeds. Both tested wild-type ecotypes started to display heat sensitivity when the shorter treatment was applied (15 min) (Figure 4.1B). Immersion at 50°C for longer periods resulted in a rapid decline on the germination rate, reaching negligible values (~10%) after treatment for 120 min. Between both wild-type ecotypes characterized, Ws seeds appear to be slightly less sensitive to heat challenge, since

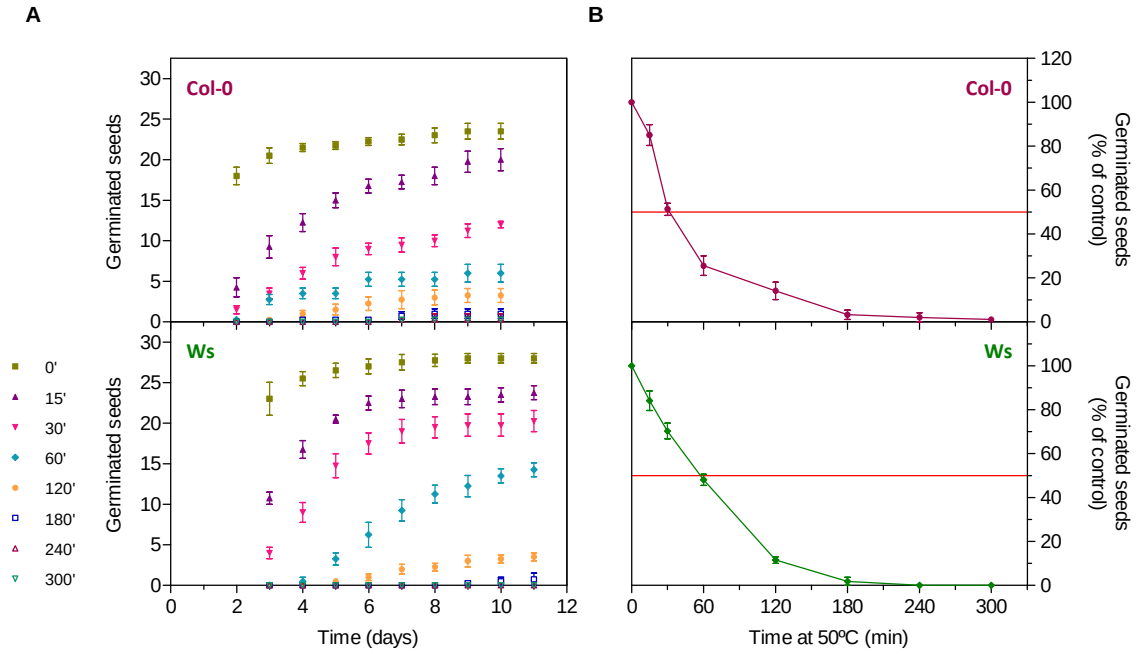

Figure 4.1. Assessment of basal thermotolerance in Col-0 and Ws wild-type seeds using a germination assay with increasing periods of heat treatment. After stratification at 4°C, seeds were treated at 50°C for different periods (15-300 min) and subsequently plated in MS agar. The number of germinated seeds was determined every day (following a period of 2-3 days) until 10-11 days upon heat stress imposition (A). Percentage of germination, in relation to untreated seed germination, was determined after 10 days upon heat stress imposition and plotted as a function of heat stress treatment period (B). Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested. Red line indicates the point where a germination rate of 50% occurs (lethal time, Lt_{50}).

incubations between 0 and 120 min produced a more moderate reduction in the germination rate. This is clearly illustrated by the higher Lt_{50} displayed by Ws seeds, determined after 10 days of seed plating, which was found to be about 60 min. In Col-0 seeds, the corresponding Lt_{50} was considered to be about 30 min. After 10 days of growth, the seedlings ensuing from stressed seeds appeared morphologically healthy and undifferentiated from untreated seeds, although being less developed due to the delayed effect on germination (Figure 4.2).

Basal thermotolerance was also evaluated by germination assays of wild-type seeds (Col-0 and Ws), using different heat-treatments (38, 41, 44, 47, 50, 53 and 56°C) (Figure 4.3). An incubation of 60 min was used for each assay, due to the previously detected moderate effect on the germination rate upon treatment at 50°C (Figure 4.1). As already noticed for the germination ability upon heat-treatment (50°C) during different periods (Figure 4.1A), the time-course evaluation of the number of germinated seeds allowed to notice a germination delay caused by the higher

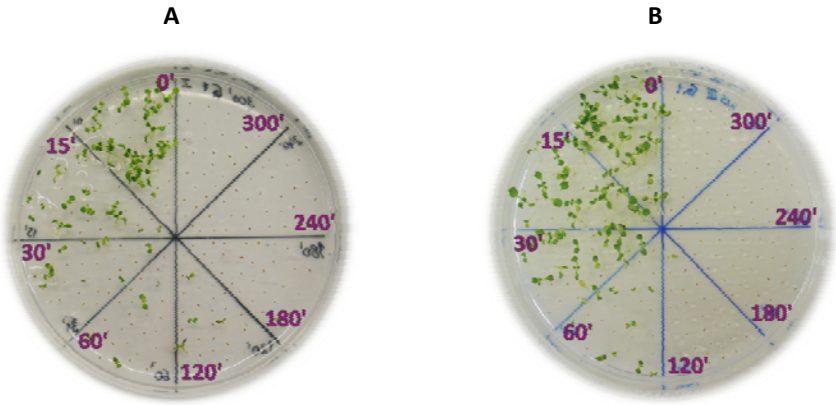

Figure 4.2. Germination assay used for determining basal thermotolerance of Col-0 (A) and Ws (B) wild-type seeds. Seeds were heat-treated (50°C) during increasing periods of treatment (15-300 min) as described in Figure 4.1 and photographs were taken 10 days after heat stress.

temperatures used for heat stress imposition (Figure 4.3A). This was particularly evident during the first 2-5 days upon heat treatment for temperatures that strongly affect germination (47 and 50°C). As already detected in the previous assay

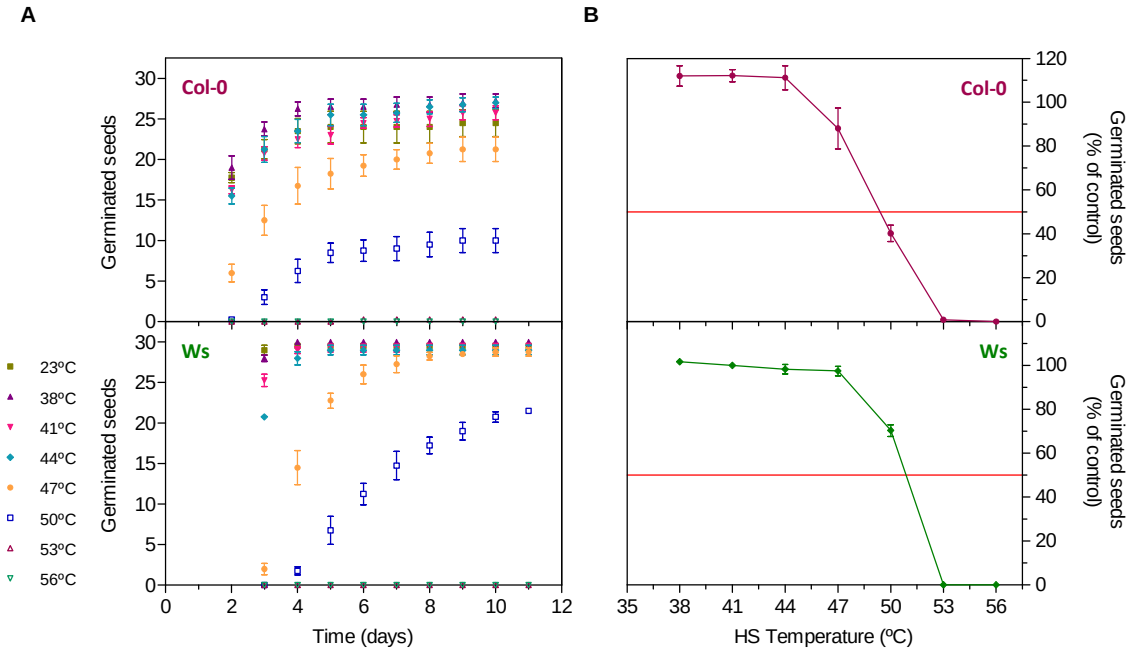

Figure 4.3. Assessment of basal thermotolerance in Col-0 and Ws wild-type seeds using a germination assay with increasing temperatures of treatment. After stratification at 4°C, seeds were treated at different temperatures (38-56°C) for 60 min and subsequently plated in MS agar. The number of germinated seeds was determined every day (following a period of 2-3 days) until 10-11 days upon heat stress imposition (A). Percentage of germination, in relation to untreated seed germination, was determined after 10 days upon heat stress imposition and plotted as a function of heat stress treatment temperature (B). Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested. Red line indicates the point where a germination rate of 50% occurs (lethal temperature, LT_{50}).

(Figure 4.2), the resultant seedlings were healthy and indistinguishable throughout the conditions analysed (Figure 4.4).

The results obtained after 10 days of seed plating show that the application of challenging temperatures below 44°C to Col-0 seeds and 47°C to Ws seeds did not strongly affect their germination (Figure 4.3B). Above these temperatures, a drastic reduction of germination rates was observed for both ecotype seeds. Similarly to the previous assay, significant germination decline was considered to occur for those heat treatments below LT_{50} (lethal temperature), corresponding to the temperature at which there is 50% of germinated seeds. The lethal temperature was determined to be between 47 and 50°C for Col-0 seeds, while Ws seeds displayed a LT_{50} between 50 and 53°C. These results suggest that Ws ecotype display higher heat tolerance than Col-0. Nevertheless, the germination was completely inhibited by a 60 min challenge at 53°C in both wild-type seeds tested.

The evaluation of basal thermotolerance by seed germination assay with variable heat treatment periods will allow to easily distinguish germination capacity alterations. If using a seed germination assay with increasing temperatures, a slight increase in the temperature (in the range of 3°C) will generate a so dramatic reduction on germination rate that will probably hinder even the small differences in the germination ability. The detection of germination alterations in *Arabidopsis* seeds under heat stress would be then facilitated by the use of variable periods of treatment.

The application of some of the previous experimental conditions to seeds, besides reducing the final germination rate, also delayed the occurrence of

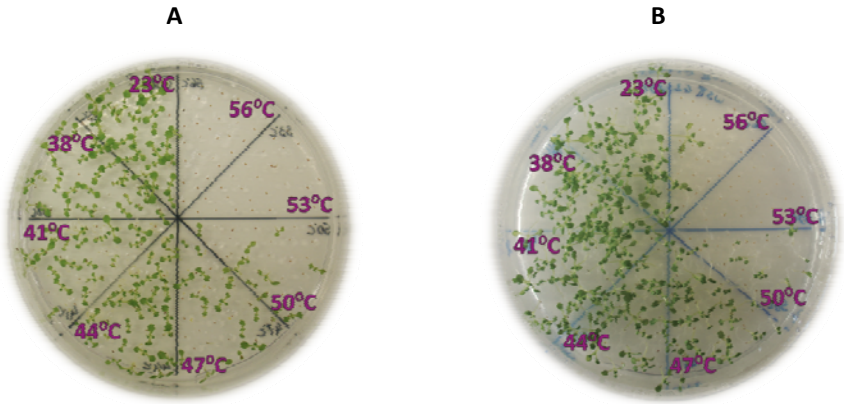

Figure 4.4. Germination assay used for determining basal thermotolerance of Col-0 (A) and Ws (B) wild-type seeds. Seeds were heat-treated with increasing temperatures of treatment (38-56°C) during 60 min as described in Figure 4.3 and photographs were taken 10 days after heat stress.

germination. Therefore, the daily determination of germinating seeds could assist in the recognition of altered timings of germination.

Basal thermotolerance evaluation by seedling survival assays

At seedling level, several processes as photosynthesis, respiration, membrane stability, level of metabolites and reactive oxygen species (ROS), along with other responses may be adversely affected by heat stress (reviewed by Wahid *et al.* 2007). The failure of these processes ultimately may lead to growth impairment and visible morphological alterations, generally manifested by the loss of green color in leaves. As suggested by Larkindale and Knight (2002), the bleaching that occurs in heat-treated (40°C, 1 h) *Arabidopsis* plants, after a recovery period in the light (3 days), may result from the oxidative damage indirectly caused by the breakdown of the photosynthetic machinery. Thus, the evaluation of thermotolerance in photosynthetically active, autotrophic seedlings can be measured in function of seedling survival after imposition of heat stress. Seedlings that remain green and actively growing are considered to be viable.

As performed in seed thermotolerance assays, two different set of conditions were employed to evaluate basal thermotolerance in 7-day-old wild-type seedlings (Col-0 and Ws). The effects of heat treatment duration (5-45 min, at 45°C) and treatment with different temperatures (40-50°C, for 20 min) were determined by the percentage of surviving seedlings, after 6 days in recovery at 23°C. According to Hong and Vierling (2000), seeds normally exhibit higher basal thermotolerance than do completely germinated seedlings. For that reason, the unchanging temperature and duration of treatment were reduced to 45°C and 20 min, respectively.

When different periods of 45°C treatment (5, 10, 15, 20, 25, 30 and 45 min) were imposed, the tested seedlings started to display heat sensitivity after 15 min (Col-0) or 20 min (Ws) (Figure 4.5). For longer treatments, the number of viable seedlings gradually reduced up to 30 min of treatment, when almost all seedlings exhibited severe injuries. The treatment period at which the survival rate would be 50% (lethal survival time, LSt_{50}) was estimated to be between 15 and 20 min for Col-0 seedlings and between 20 and 25 min for Ws ecotype. Altogether, the results suggest that Ws seedlings are less sensitive to heat than Col-0.

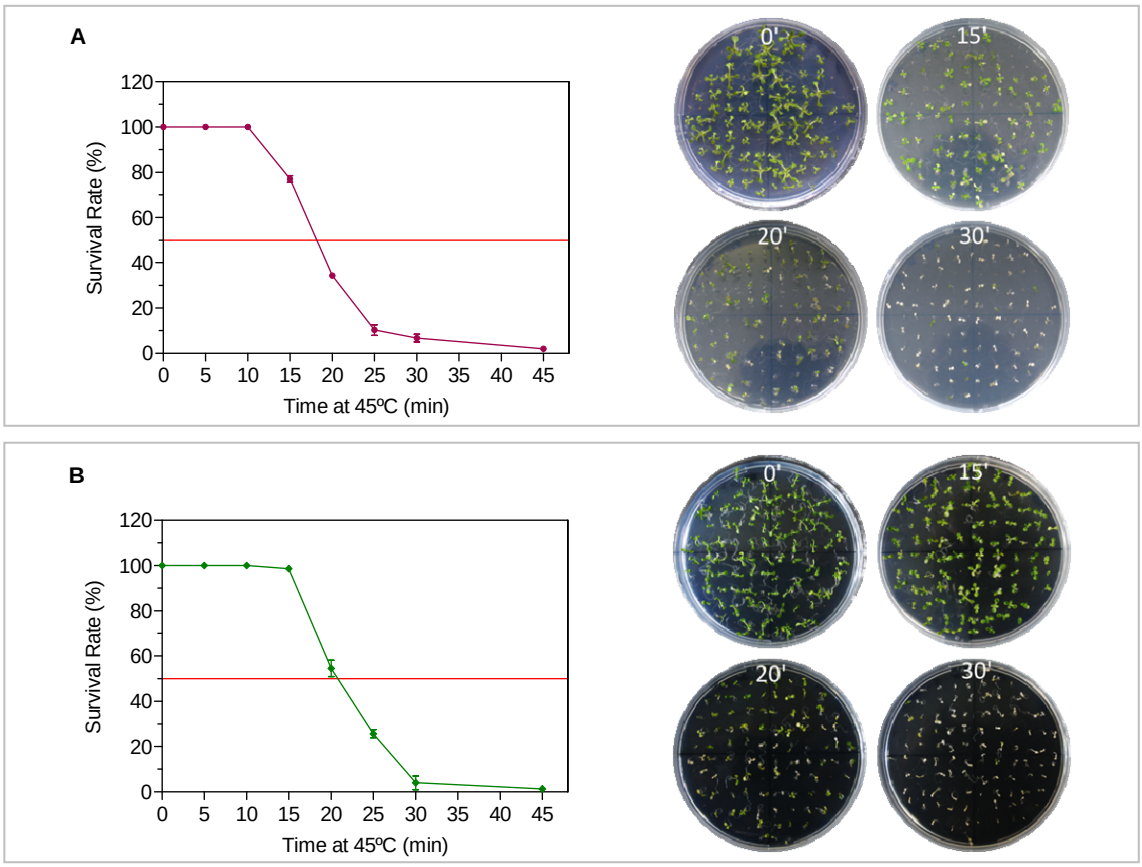

Figure 4.5. Assessment of basal thermotolerance in Col-0 (A) and Ws (B) wild-type seedlings using a seedling survival assay with increasing periods of heat treatment. Seven-day-old seedlings grown in MS agar were treated at 45°C for different periods (5-45 min) followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Photographs were taken 6 days after heat stress. Mean and SEM were derived from counting in 3 replicates in the same condition, all containing 100 seeds of each genotype tested. Red line indicates the point where a survival rate of 50% occurs (lethal survival time, LSt_{50}).

A similar basal thermotolerance response was obtained, when dissimilar temperatures (40, 42.5, 45, 47.5 and 50°C) of treatment were applied for 20 min (Figure 4.6). Seedlings (Col-0 and Ws) did not display heat sensitivity up to 42.5°C-heat stress. But, when seedlings were treated at 45°C for 20 min and then returned to standard conditions at 23°C, a progressive bleaching was visible over 6 days in a large percentage of seedlings. Although both ecotypes were significantly affected, Col-0 seedlings displayed the most severe reaction. Only 32% of the treated Col-0 seedlings remained viable, against 56% of the Ws ecotype. Accordingly, LST_{50} (lethal survival temperature; temperature at which the survival rate would be 50%) was lower for Col-0 (between 42.5 and 45°C) when compared to Ws seedlings (between 45 and 47.5°C). These results are in accordance with those from survival assays upon a 45°C-

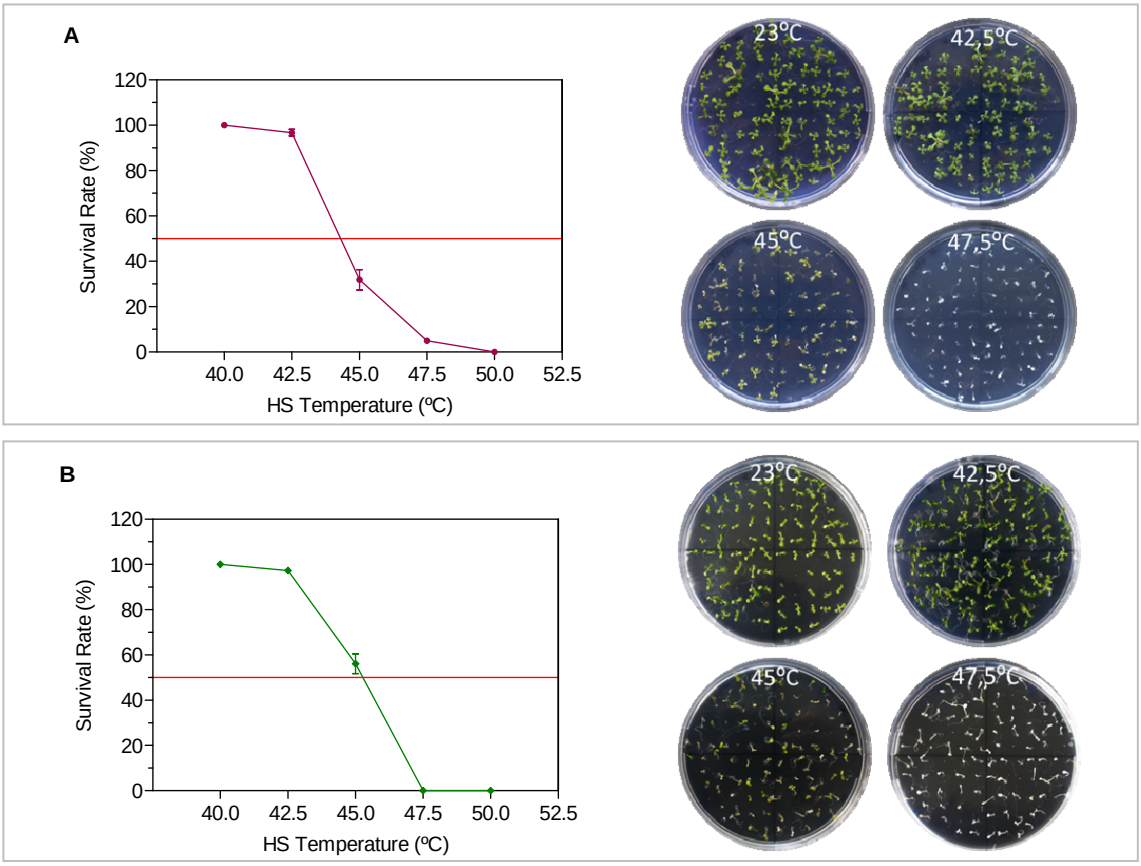

Figure 4.6. Assessment of basal thermotolerance in Col-0 (A) and Ws (B) wild-type seedlings using a seedling survival assay with increasing temperatures of treatment. Seven-day-old seedlings grown in MS agar were treated at different temperatures (40-50°C) for 20 min followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Photographs were taken 6 days after heat stress. Mean and SEM were derived from counting in 3 replicates in the same condition, all containing 100 seeds of each genotype tested. Red line indicates the point where a survival rate of 50% occurs (lethal survival temperature, LST_{50}).

heat treatment for different periods, in which Ws also displayed more thermotolerance than Col-0 (Figure 4.5).

As verified for germination assays, increasing periods of treatment revealed a more moderate decrease in survival of treated seedlings, as compared to increasing temperatures of treatment. Therefore, variable periods of heat treatment seem to be more accurate for determining slight basal thermotolerance alterations both in seed and seedling growth stages.

Acquired thermotolerance evaluation by seedling survival assays

A conditioning pre-treatment with moderate temperatures prior to a lethal temperature treatment has been shown to partially improve survival in several plant species (reviewed by Larkindale *et al.* 2005*b*). This phenomenon, known as acquired

thermotolerance, enables plants to withstand excessively high temperatures that would be damaging or lethal without such acclimation treatment (Vierling 1991). The evaluation of acquired thermotolerance in *Arabidopsis* is crucial to establish a proper procedure for isolation of acquired thermotolerance mutants. The temperature at which thermotolerance is acquired in *Arabidopsis* was assessed by incubating wild-type 7-day-old seedlings (Col-0 and Ws) at different temperatures (30, 32, 34, 36, 38 and 40°C) for 60 min, followed by 120 min at 23°C, prior to a heat treatment (45°C) for 20 min. The challenging treatment was defined accordingly to the results presented in Figure 4.5 and 4.6. Six days after a 45°C treatment during 20 min, only about 32% of Col-0 seedlings and about 55% of Ws seedlings survived. When seedlings were pre-incubated at 30°C, a higher tolerance to subsequent heat exposure was detected (Figure 4.7). This enhanced survival rate progressively increased with higher pre-conditioning temperature incubations. Six days upon heat stress imposition, the seedlings acclimated with higher temperatures appeared to be more developed than controls, which can be also seen as an indicator of enhanced thermotolerance capacity. The highest levels of thermal protection, corresponding to 100% of survival, were attained after pre-incubation at 34°C for Col-0 and 32°C for Ws seedlings. These results are in agreement with the suggestion that Ws displays a higher thermotolerance in relation to Col-0 ecotype. However, this conclusion should be taken with care, since the results obtained without acclimation period are different in both ecotypes. From these results, using the predetermined experimental conditions (heat treatment of 45°C for 20 min, after a recovery period of 120 min at 23°C), it seems reasonable to suggest the application of 60 min acclimation temperatures of 30°C up to 34°C for the identification of phenotypes in mutants defective in acquiring thermotolerance.

TESTING PROTOCOLS FEASIBILITY - PHENOTYPIC ANALYSIS OF HEAT-ASSOCIATED MUTANTS

The characterization of basal and acquired thermotolerance in *Arabidopsis* is crucial to establish a proper procedure for isolation of mutants defective in both processes. Using the conditions previously established for germination and survival

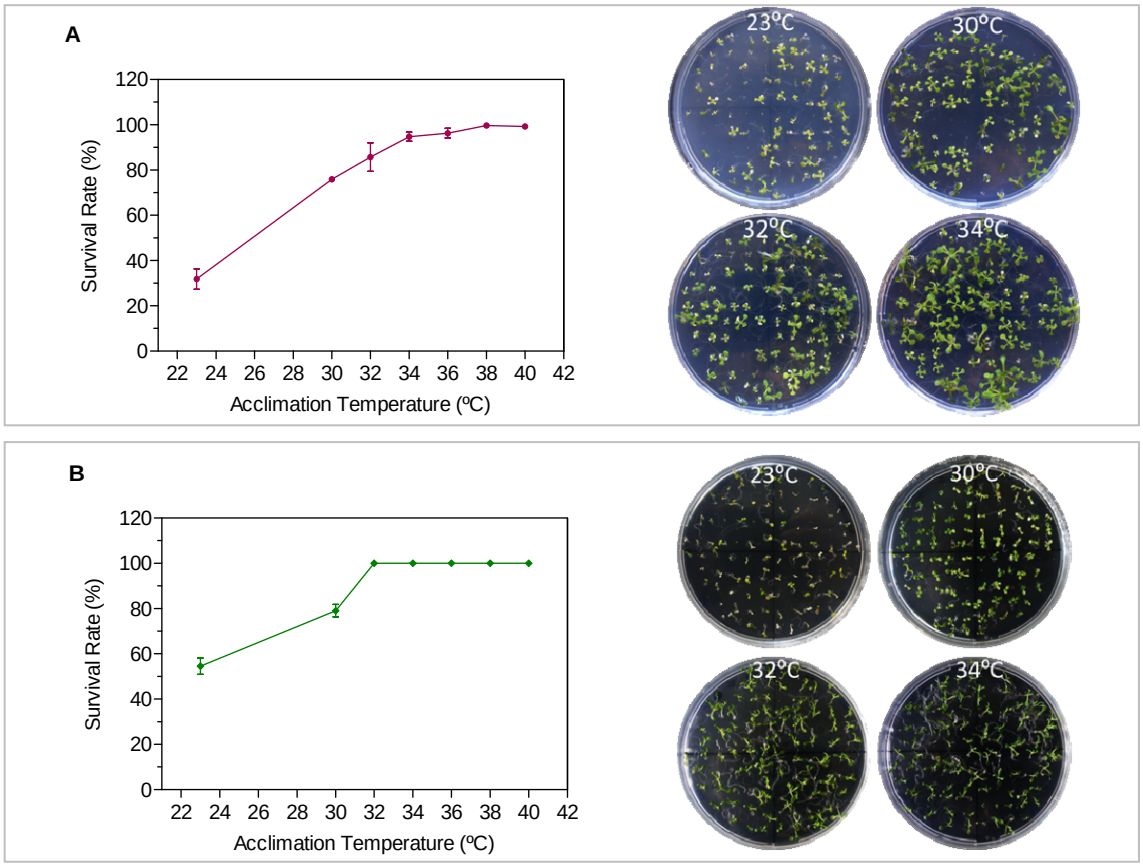

Figure 4.7. Assessment of acquired thermotolerance in Col-0 (A) and Ws (B) wild-type seedlings using a seedling survival assay with increasing acclimation temperatures. Seven-day-old seedlings grown in MS agar were pre-conditioned at different temperatures (30-40°C) for 60 min and subsequently allowed to recover at 23°C for 120 min. Seedlings were then further treated at 45°C for 20 min followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Photographs were taken 6 days after heat stress. Mean and SEM were derived from counting in 3 replicates in the same condition, all containing 100 seeds of each genotype tested.

assays (temperature and duration of heat treatment, as well as acclimation temperature), two *Arabidopsis* mutants were tested for basal and acquired thermotolerance at seed and seedling levels. These T-DNA insertion mutants (generated in Col-0 ecotype) were previously described as being disrupted in processes strongly associated with heat responses, specifically in heat shock protein (HSP) synthesis (Hong and Vierling 2000; 2001) and ROS signaling (Dat *et al.* 1998; Larkindale and Huang 2004). In addition, both mutants were previously found to have temperature-related phenotypes (Larkindale *et al.* 2005*a*).

One of the chosen mutants, *hot1-3*, has an insertion in the second exon of the heat shock protein 101 gene (At1g74310) and is null for HSP101 protein expression (Hong and Vierling 2001). As reviewed by Hong *et al.* (2003), based on the analogy with yeast and bacteria systems, HSP101 seems to play a role as a chaperone ATPase

involved in dissolution of cytosolic or nuclear protein aggregates formed during heat stress. Studies on germination, growth and development of *hot1-3* mutant plants under standard conditions demonstrated that HSP101 activity is not essential in the absence of stress (Hong and Vierling 2001). In opposition, when submitted to high-temperature stress (45°C for 120 min), *hot1-3* seedlings exhibited a thermotolerance defect either without or with a pre-adaptation period (38°C for 90 min, followed by 2 h at 22°C). A significantly reduced basal thermotolerance was also observed in *hot1-3* seeds, after treatment at 45°C for 90-150 min, as evaluated by hypocotyl elongation.

The other selected mutant, *atrbohD*, has an insertion in the fifth exon of the NADPH/respiratory burst oxidase homolog gene (At5g47910; Torres *et al.* 1998; 2002). *Arabidopsis* encodes ten respiratory burst oxidase homologs (*Atrboh* genes), which have been proposed to play a role in the production of ROS (Torres *et al.* 2002). The generation of ROS has already been recognized to act in several complex signaling networks, including abiotic stress responses (reviewed by Suzuki and Mittler 2006). Accordingly, the induction of stress-associated pathways and defense mechanisms are thought to be regulated through signal transduction events mediated by ROS. Genetic evidences suggested that *atrbohD* encodes a probable component of a NADPH oxidase responsible for the generation of ROS (Torres *et al.* 2002) and seems to be indirectly involved in the regulation of abiotic stress responses (Torres and Dangl 2005). Phenotypic characterization of *atrbohD* 4-week-old rosettes under normal conditions revealed no significant morphological alterations, although they seemed to be slightly smaller when compared to wild-type Col-0 plants. In response to heat stress, *atrbohD* showed a moderate defect in basal and acquired thermotolerance of 7-day-old seedlings but no phenotype was significantly found for basal seed thermotolerance (Larkindale *et al.* 2005*a*). When compared to *hot1-3*, which is strongly affected by temperature, *atrbohD* presents a moderate heat phenotype.

To demonstrate the effectiveness of the described assays for identification of phenotypic alterations in *Arabidopsis* thermotolerance mutants, a detailed characterization of *hot1-3* and *atrbohD* mutant lines was performed. In order to achieve feasible results, wild-type (Col-0) and mutant lines were grown at the same time for synchronized seed production. Although the characterization of Col-0 thermotolerance was initially accomplished individually (Figure 4.1, 4.3, 4.5A, 4.6A and

4.7A), this line was always included in the following assays to serve as a wild-type control subjected to the same conditions.

Validating germination assays

Using the same parameters previously defined for the characterization of basal thermotolerance in wild-type seeds, *hot1-3* and *atrbohD* were evaluated for defects in seed germination. Mutant seeds tested with different periods (15, 30, 60, 120, 180, 240 and 300 min) at 50°C were differentially affected by heat stress (Figure 4.8). When comparing to the response curve of Col-0, *hot1-3* seeds showed to be highly defective in seed germination. A strong reduction in germination was revealed by *hot1-3* seeds with just 15 min of heat treatment. Germination rate abruptly declined when longer periods at 50°C were employed and was completely prevented by a 120 min challenge. A different response was obtained for *atrbohD* seeds, which behaved similarly to wild-type seeds when exposed to similar heat treatment. An exception was observed after a 120 min treatment at 50°C, which produced a significant reduction in germination as compared to wild-type seeds. The distinct sensitivity to heat treatment displayed by the mutant lines was also demonstrated by analysis of the number of germinated seeds throughout the following 9 days after treatment (Figure 4.9). Additionally to a reduced germination rate, treatment at 50°C for 15-60 min produced a delayed effect on germination of *hot1-3* seeds (Figure 4.9B) but not of *atrbohD* seeds (Figure 4.9C).

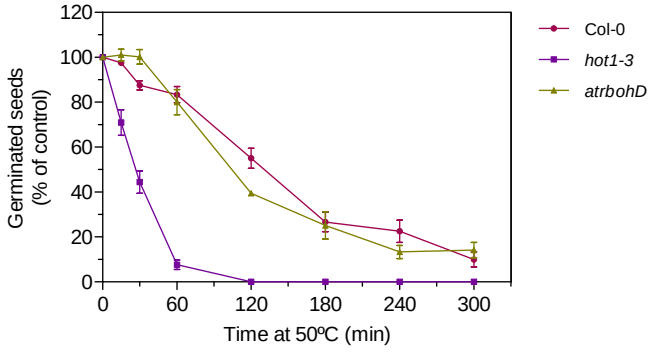

Figure 4.8. Assessment of basal thermotolerance in Col-0 wild-type, *hot1-3* and *atrbohD* mutant seeds using a germination assay with increasing periods of heat treatment. After stratification at 4°C, seeds were treated at 50°C for different periods (15-300 min) and subsequently plated in MS agar. Percentage of germination, in relation to untreated seed germination, was determined after 9 days upon heat stress imposition and plotted as a function of heat stress treatment period. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

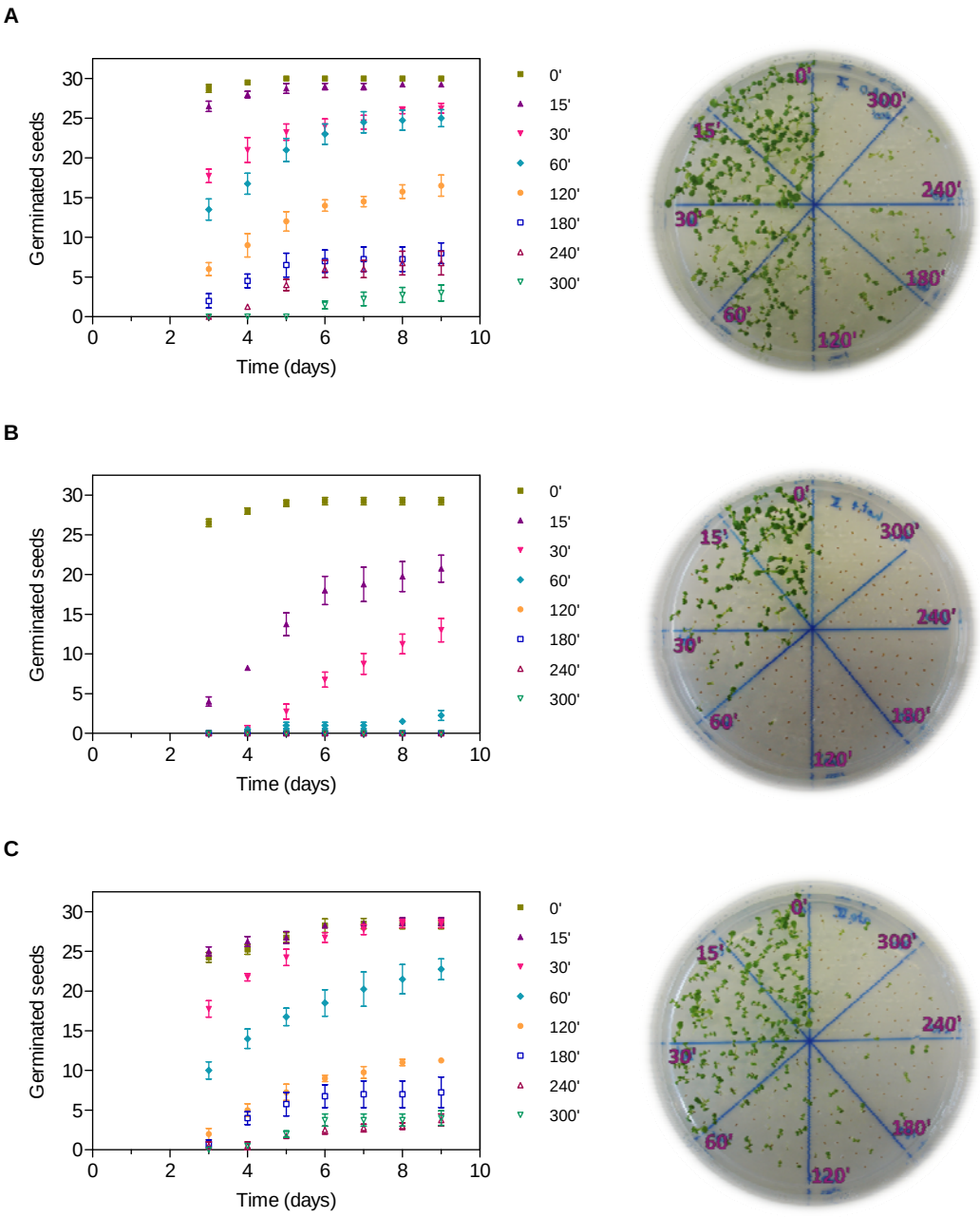

Figure 4.9. Germination time-course of Col-0 wild-type (A), *hot1-3* (B) and *atrbohD* (C) mutant seeds after heat stress at 50°C for different periods (15-300 min). Seeds were heat-treated as described in Figure 4.8 and the number of germinated seeds was determined every day (following a period of 3 days after treatment) until 9 days upon heat stress imposition. Photographs were taken 9 days after heat stress. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

When seeds were tested for basal thermotolerance in germination assays performed with different temperatures (43, 46, 49, 52 and 55°C) for 60 min, *hot1-3* seeds displayed a more severe response to heat treatment than wild-type or *atrbohD* seeds (Figure 4.10). According to the results presented in Figure 4.3, where challenging

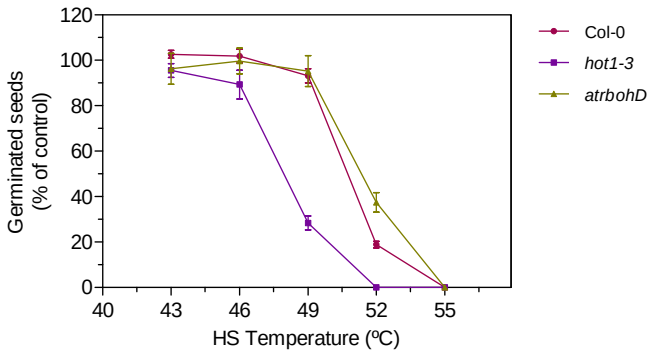

Figure 4.10. Assessment of basal thermotolerance in Col-0 wild-type, *hot1-3* and *atrbohD* mutant seeds using a germination assay with increasing temperatures of treatment. After stratification at 4°C, seeds were treated at different temperatures (43-55°C) for 60 min and subsequently plated in MS agar. Percentage of germination, in relation to untreated seed germination, was determined after 9 days upon heat stress imposition and plotted as a function of heat stress treatment temperature. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

temperatures of 38-44°C did not cause a deleterious effect on germination rate of Col-0 seeds, the tested temperature range was modified (43-55°C). Germination of *hot1-3* seeds was affected at a challenge temperature of 46°C and declined until total hindrance at 52°C. Between this array of temperatures (46-52°C), *hot1-3* response curve was significantly different from Col-0 and *atrbohD* seeds. Both Col-0 and *atrbohD* seeds were able to tolerate high temperatures (up to 49°C) without suffering a reduction in germination rate. Although *atrbohD* seeds exposed to a 52°C-treatment displayed a higher level of germination (37.5%) in relation to Col-0 (18.9%), this value was not statistically different. As previously determined, a delay effect on germination of *hot1-3* seeds was also evident for treatments at 46°C and 49°C (Figure 4.11).

As observed in the characterization of Col-0 basal thermotolerance, mutant seeds which were able to germinate after heat treatment produced healthy growing seedlings undifferentiated from those originated from untreated (0 min or 23°C treatments) or wild-type seeds (Figures 4.9 and 4.11).

Basal seed thermotolerance of *hot1-3* and *atrbohD* mutants was previously analyzed after treatment at 45°C for 220 min by germination assessment (Larkindale *et al.* 2005*a*). While only 9.1% of *hot1-3* treated seeds were able to germinate, seed germination in *atrbohD* mutant (76.8%) was not statistically different from wild-type seeds (93.4%). These results are in accordance with those presented in Figure 4.8 and 4.10, although quite different conditions were employed. A similar percentage of

germination was observed when *hot1-3* seeds were treated at a higher temperature (50°C) but only for 60 min (Figure 4.8). When a temperature close to the used for Larkindale *et al.* (2005*a*) was applied, although during a shorter period (46°C, 60 min), the reduction in *hot1-3* germination was moderate (Figure 4.10). For *atrbohD* mutant,

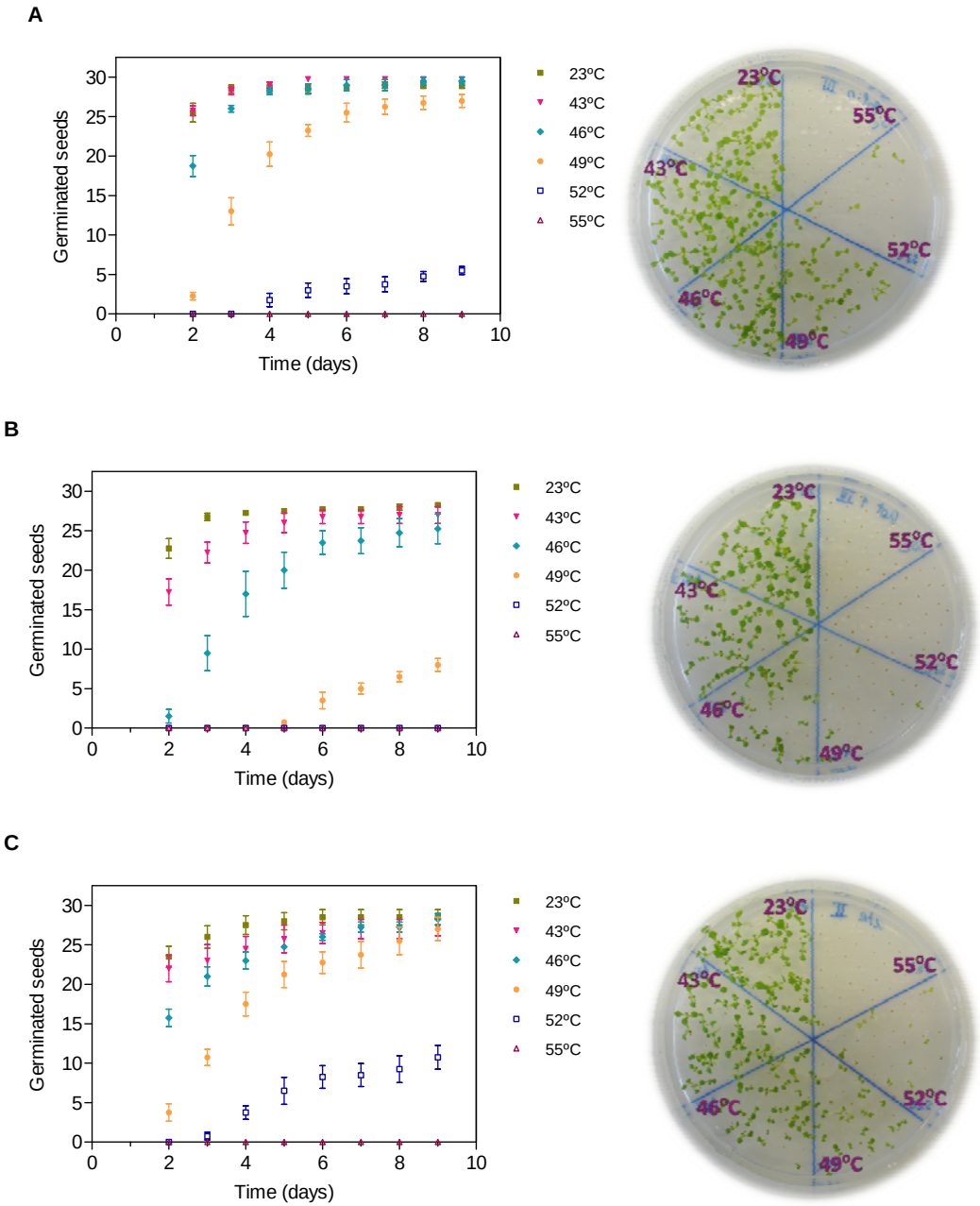

Figure 4.11. Germination time-course of Col-0 wild-type (A), *hot1-3* (B) and *atrbohD* (C) mutant seeds after heat stress at different temperatures (43-55°C) for 60 min. Seeds were heat-treated as described in Figure 4.10 and the number of germinated seeds was determined every day (following a period of 2 days after treatment) until 9 days upon heat stress imposition. Photographs were taken 9 days after heat stress. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

a significant reduction in germination was only observed after treatment at 50°C for 120 min (Figure 4.8). This result was not detected in the assays performed by Larkindale *et al.* (2005*a*).

Validating seedling survival assays

Assessment of basal thermotolerance defects in seedling survival was performed for the selected mutant lines, using the predetermined experimental conditions. Treatment at 45°C for different periods (10, 15, 20, 25, 30 and 45 min) revealed different levels of thermosensitivity of mutant 7-day-old seedlings (Figure 4.12). Six days after heat stress imposition at 45°C for 20 min, Col-0, *hot1-3* and *atrbohD* seedlings displayed statistically different survival rates of 67.3%, 12.5% and 52.9%, respectively. Therefore, *hot1-3* seedlings were extremely affected by heat treatment, while *atrbohD* seedlings exhibited a more moderate phenotype. Considering the period of heat treatment, only the 20 min challenge has successfully

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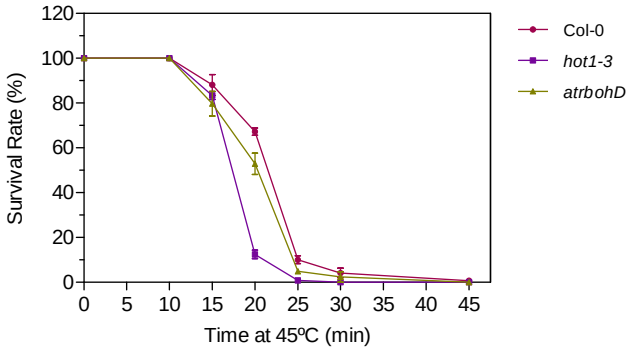

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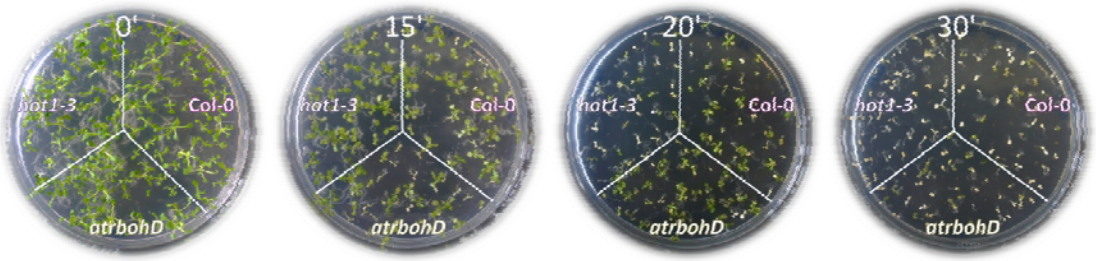

Figure 4.12. Assessment of basal thermotolerance in Col-0 wild-type, *hot1-3* and *atrbohD* mutant seedlings using a seedling survival assay with increasing periods of treatment. Seven-day-old seedlings grown in MS agar plates were treated at 45°C for different periods (10-45 min) followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival (A). Photographs were taken 6 days after heat stress (B). Mean and SEM were derived from measurements in 3 replicates in the same condition, all containing 100 seeds of each genotype tested.

allowed to identify significant heat-associated phenotypic alterations in both mutant seedlings.

Similar results were obtained for basal thermotolerance of mutant 7-day-old seedlings when using the survival assay with different temperatures (40, 42.5, 45, 47.5 and 50°C) for 20 min (Figure 4.13). After a period of 6 days in recovery (23°C) from a 20 min challenge at 45°C, survival rates of Col-0, *hot1-3* and *atrbohD* seedlings were 64.4%, 8.7% and 49.9%, respectively. Therefore, mutant seedlings showed dissimilar degrees of basal thermotolerance, which was much lower in *hot1-3* seedlings than in *atrbohD* seedlings. Only one of the experimental conditions analysed (45°C, 20 min) was effective for recognition of significant altered thermotolerance responses in *hot1-3* and *atrbohD* seedlings.

The results presented are in agreement with the previous conclusions of Larkindale *et al.* (2005*a*). Seven-day-old seedlings of *hot1-3* and *atrbohD* mutants heat-

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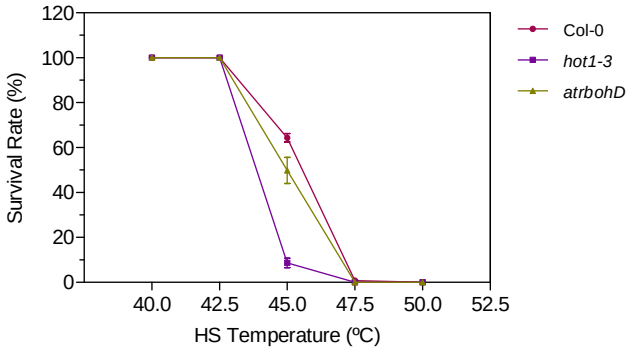

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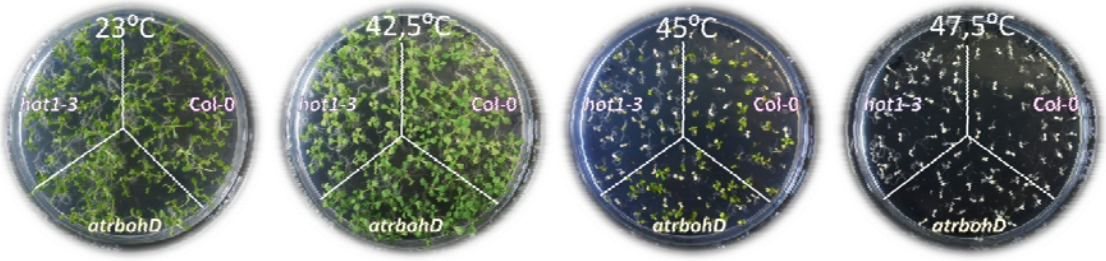

Figure 4.13. Assessment of basal thermotolerance in Col-0 wild-type, *hot1-3* and *atrbohD* mutant seedlings using a seedling survival assay with increasing temperatures of treatment. Seven-day-old seedlings grown in MS agar plates were treated at different temperatures (40-50°C) for 20 min followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Photographs were taken 6 days after heat stress. Mean and SEM were derived from measurements in 3 replicates in the same condition, all containing 100 seeds of each genotype tested.

treated at 45°C for 60 min registered reduced basal thermotolerance (approximately 10 and 40% of wild-type, respectively). While *hot1-3* was considered to have a severe defect on basal thermotolerance, *atrbohD* seedlings were moderately affected. Accordingly, as shown in Figure 4.12 and 4.13, *hot1-3* was more severely affected than *atrbohD*. Although shorter lengths of 45°C-treatment were employed (10-45 min), the results obtained in both experiments can be related since the treatments performed by Larkindale *et al.* (2005*a*) were applied in an incubator and not by immersion into a water bath.

The effect of diverse acclimation temperatures (30, 32, 34, 36 and 38°C) in providing thermal protection to the selected *Arabidopsis* mutant seedlings was evaluated (Figure 4.14). Seven-day-old seedlings were tested for acquired thermotolerance by pre- incubation at diverse temperatures for 60 min, followed by 120 min at 23°C and heat treatment of 45°C for 20 min. Six days after heat treatment

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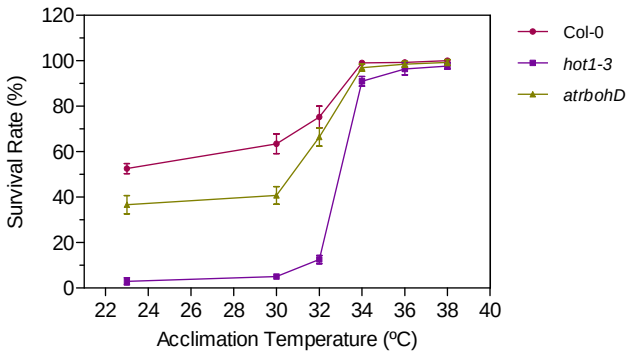

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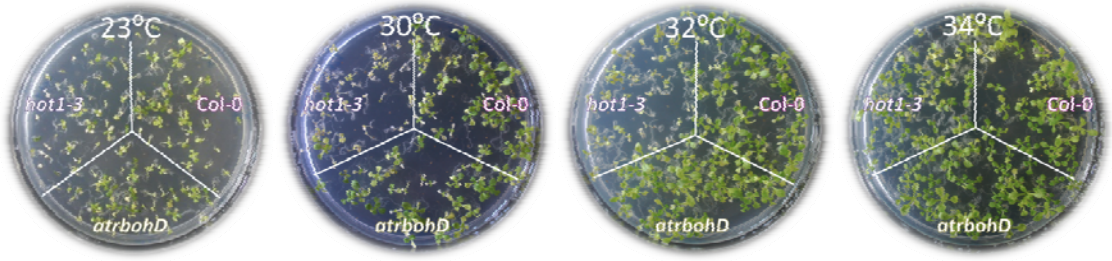

Figure 4.14. Assessment of acquired thermotolerance in Col-0 wild-type, *hot1-3* and *atrbohD* mutant seedlings using a seedling survival assay with increasing acclimation temperatures. Seven-day-old seedlings grown in MS agar plates were pre-conditioned at different temperatures (30-38°C) for 60 min and subsequently allowed to recover at 23°C for 120 min. Seedlings were then further treated at 45°C for 20 min followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Photographs were taken 6 days after heat stress. Mean and SEM were derived from measurements in 3 replicates in the same condition, all containing 100 seeds of each genotype tested.

the percentage of surviving seedlings was estimated. At lower acclimation temperatures (30 and 32°C), both mutants showed statistically different reductions in acquired thermotolerance. A reduced acquired thermotolerance was observed in *hot1-3* mutant, which presented dramatically reduced ability to recover from heat challenge (<15% survival rate), when acclimated at these temperatures. In the same conditions, *atrbohD* seedlings displayed higher survival rates: ~40% and ~66% for acclimation temperatures of 30 and 32°C, respectively. This distinct heat sensitivity was already noted by Larkindale *et al.* (2005*a*). The highest levels of thermal protection for both mutants and wild-type seedlings were achieved after a pre-conditioning treatment at 34°C. No significant differences in survival rate were found for acclimation temperatures of 34°C or above as compared to Col-0 plants.

CONCLUSIONS

Several phenotypic assays were developed and used for detection of thermotolerance defects in *Arabidopsis* mutants at seed and 7-day-old seedlings. The protocols described are useful to launch the hunting for phenotypic alterations in mutants that are deficient either in basal or acquired thermotolerance or in both. Basal thermotolerance assays were based in evaluation of seed germination and seedling survival after heat treatment. A series of experimental conditions were proposed to be tested using: (i) variable periods of immersion into a fixed temperature water bath or (ii) variable temperatures during an unchanging period of treatment. The range of conditions employed pretended to include weak treatments that did not produce any effect on germination/seedling survival, as well as severe conditions that were lethal to seeds/seedlings. Acquired thermotolerance assay was solely based in assessment of seedling survival. In this assay, variable preconditioning temperatures were tested for their capacity in providing minimum and maximal thermal protection to seedlings further exposed to a more challenging treatment.

The proposed procedure was used for characterization of basal and acquired thermotolerance in wild-type (Col-0 and Ws) seeds and seedlings. By accomplishing the thermal responses in wild-type lines we were able to determine the array of treatment conditions that would probably result in the detection of heat phenotypes in mutants. Selected treatments were delimited by the conditions that produce the weaker and the strongest effects on germination and seedling survival. For recognition of basal thermotolerance defects, evaluation of seed germination should be performed after treatment at 50°C for 15-180 min or at 47-53°C for 60 min. For detection of slight germination alterations a protocol with different periods (15-180 min) of heat imposition at a fixed temperature (50°C) would be preferred, since the differences detected in each treatment were more subtle than those detected with a protocol where increasing temperatures (47-53°C) are imposed during a defined period (60 min).

When using a limiting heat temperature, a delayed effect on germination progression was always observed in all wild-type ecotypes. Furthermore, when germinating seeds were scored during the subsequent days after heat treatment,

longer delays were observed as stronger treatments were applied. This result suggests that seeds exhibited an outstanding capacity to recover from heat treatment by prolonging the dormant state. Therefore, when searching for a heat-related phenotype, a careful examination of the time-course of germination should be performed since the failure of viable treated seeds to germinate during the same period observed in control seeds can be considered a phenotype by itself. For the same reason, when comparing germination rates between different experiments and/or mutants, special attention should be paid to the assessment day used for the calculations.

Identification of altered basal thermotolerance by seedling survival assays should be carried out by immersion at 45°C for 15-30 min or at 42.5-47.5°C for 20 min. Once more, increasing temperatures with a fixed period of treatment (20 min) were found to promote more drastic differences in plant responses than defining the temperature and increasing treatment periods. In order to test the ability to acquire thermotolerance, a seedling survival assay should be performed with acclimation treatment at 30-34°C for 1 h, plus 120 min at 23°C, followed by the challenge heat treatment of 45°C for 20 min.

Using the same time and temperature parameters, the predefined assays successfully detected heat phenotypes similar to those already reported for *hot1-3* and *atrbohD* mutants (Hong and Vierling 2001; Larkindale *et al.* 2005*a*). A severe defect in basal thermotolerance (at seed and seedling growth stages) as well as in acquired thermotolerance was found for *hot1-3* mutant. A more moderate although still significant phenotype was observed for *atrbohD* mutant seedlings in basal and acquired thermotolerance assays. Although *atrbohD* seeds did not show a significant phenotype in the germination assay previously performed by Larkindale *et al.* (2005*a*), we were able to detect a significant difference, mainly after treatment at 50°C for 120 min as compared to Col-0 germination. Therefore, our results suggest that *atrbohD* mutant may be defective in basal seed thermotolerance as well.

In all thermotolerance assays performed with *hot1-3* and *atrbohD* mutants, the heat phenotypes identified were observed in the conditions predetermined as most favorable for detection of thermotolerance defects. As a conclusion, the outlined protocols proved to be effective for detection of phenotypic alterations in mutants

disrupted in processes associated with heat responses. Each one of the proposed assays embraces a large series of experimental conditions, which made them particularly helpful as an inexpensive and undemanding initial approach.

When comparing the characteristics of thermotolerance assessed initially for Col-0 ecotype (Figures 4.1 to 4.7) with those obtained for the same ecotype together with mutants (Figures 4.8 to 4.14), some inconsistency was observed in the response curves. The first pool of Col-0 seeds was apparently less tolerant to the heat treatments applied. Two essential remarks must be considered: (i) the synchronization of all tested plants is important to guarantee that similar conditions are used for seed production and assure equal circumstances for all lines in the subsequent thermotolerance assays; (ii) although secondary parameters like humidity and light intensity are controlled as much as possible, there is no warranty that the conditions gathered inside the plates during treatment, as well as in the subsequent period at 23°C, can be exactly reproduced in later assays. For these reasons, the wild-type control should always be included in the assays to assure the most feasible phenotypic evaluation.

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Chapter 5

Functional analysis of an *Arabidopsis thaliana* RING finger protein (HZF) induced by heat stress

CONTENTS

Functional analysis of an *Arabidopsis thaliana* RING finger protein (HZF) induced by heat stress

ABSTRACT

Functional genomics currently provide tools for facilitating the functional assignment of yet uncharacterized genes with a particular role for thermotolerance. The transcriptome analysis of heat-stressed *Arabidopsis thaliana* plants performed using Affymetrix microarray data led to the identification of *HZF*, a heat-induced functionally uncharacterized gene encoding a putative zinc finger family protein. Structural characterization of *HZF* was performed by *in silico* analysis, namely based on structure information of gene and protein, sequence homology, co-expressed gene network and *HZF* expression pattern during normal development of *Arabidopsis* wild-type plants and seed germination. HZF was found to have a conserved C3H2C3-type RING domain, highly similar to those from other uncharacterized *Arabidopsis* RING finger-containing proteins. The presence of this domain, together with the high degree of protein similarity with CIP8, suggested that HZF may have E3 ubiquitin ligase activity. Aiming to assign a biological function to HZF and determine its particular role in heat stress tolerance, a homozygous T-DNA insertion mutant line (*hzf*) was successfully isolated through diagnostic PCR from publicly available heterogeneous seeds stock. Phenotypic analysis of loss-of-function *hzf* mutants revealed delayed germination ability in the following days after heat treatment when compared to wild-type plants. Despite the gene redundancy that almost certainly occurs between HZF and its most similar gene product (L-HZF), the delayed germination seems to be the result of impaired ubiquitination of a regulator protein implicated in promoting seed dormancy or repressing germination. The evaluation of *HZF* transcripts in heat-treated (38°C for 1 h) wild-type seedlings showed strongest induction 15 min after treatment, which confirmed the involvement of HZF in the initial phase of heat stress response. A dual function of HZF in degrading a key regulator protein controlling dormancy/germination processes and HSF signaling was then suggested. Several *HZF*-based expression vectors for overexpression studies and *in situ* analysis were constructed and used to transform wild-type *Arabidopsis* plants. The resultant transgenic T3 plants will be soon available to proceed in the functional characterization of *HZF*.

KEYWORDS *Arabidopsis thaliana* · C3H2C3-type RING finger · E3 ubiquitin ligase · Germination/dormancy phenotype · HZF functional characterization · Thermotolerance

ABBREVIATIONS aa: **a**mino **a**cid · ABA: **ab**scisic **a**cid · ABRC: ***A**rabidopsis **B**iological **R**esource **C**enter* · AGRIS: ***A**rabidopsis **G**ene **R**egulatory **I**nformation **S**ervice* · APC: **a**naphase-**p**romoting **c**omplex · ARAMEMNON: *Plant Membrane Protein Database* · AtcisDB: ***A**rabidopsis **t**haliana **cis**-regulatory **D**atab**a**se* · BAR: ***B**io-**A**rray **R**esource* · BLAST: ***B**asic **L**ocal **A**lignment **S**earch **T**ool* · bp: **b**ase **p**air · CIP8: **C**OP1-**i**nteracting **p**rotein **8** · COP1: **co**nstitutive **p**hotomorphogenic **1** protein · DMSO: **di**m**e**thyl **s**ulf**o**xide · DNA: **d**eoxyribo**n**ucleic **a**cid · eFP: **e**lectronic **F**luorescent **P**ictographic · *g*: **g**ravitational acceleration · GFP: **g**reen **f**luorescent **p**rotein · GUS: β-**g**luc**u**ronida**s**e · HECT: **h**omology to **E**6-AP**C**-**t**erminus · HSF: **h**eat **s**hock transcription **f**actor · HSP: **h**eat **s**hock **p**rotein · HZF: **h**eat **z**inc **f**inger family protein · INRA: ***I**nstitut **N**ational de la **R**echerche **A**gronomique* · kDa: **k**ilo**da**ltons · LB: **L**uria-**B**ertani · L-HZF: **l**ike **HZF** · mRNA: **m**essenger **RNA** · MS: **M**urashige and **S**koog · NASC: ***N**ottingham **A**rabidopsis **S**tock **C**enter* · NCBI: ***N**ational **C**enter for **B**iotechnology **I**nformation* · NLS: **n**uclear **l**ocalization **s**ignal · ORF: **o**pen **r**eading **f**rame · PCR: **p**olymerase **c**hain **r**eaction · PEG: **p**oly**e**thylene **g**lycol · PRT1: **p**ro**t**eolysis 1 · RING: **R**eally **I**nteresting **N**ew **G**ene · RNA: **r**ibo**n**ucleic **a**cid · RNAi: **RNA i**nterference · rpm: **r**otation **p**er **m**inute · SCF: **S**KP1, **C**DC53 and **F**-box containing protein · *SEM*: **s**imple and **e**fficient **m**ethod of *E. coli* transformation · SEM: **s**tandard **e**rror of the **m**ean · SIGnAL: ***S**alk **I**nstitute **Gen**omic **A**nalysis **L**aboratory* · SOB: **s**uper **o**ptimal **b**roth · SOC: **s**uper **o**ptimal broth with **c**atabolite repression · TAIR: ***T**he **A**rabidopsis **I**nformation **R**esource* · TB: **t**ransformation **b**uffer · T-DNA: **t**ransferred **DNA** · TIGR: ***T**he **I**nstitute for **G**enomic **R**esearch* · UTR: **u**n**t**ranslated **r**egion · Ws: *A. thaliana* **W**assilew**s**kija ecotype

INTRODUCTION

In 2000, plant science community established the challenging goal of determining the function of all genes in the model plant *Arabidopsis thaliana* by the year 2010 ("2010 Project"; Chory *et al.* 2000). To achieve this purpose, the functional annotation of individual genes relies on several experimental strategies, such as mutant analysis, RNA expression or protein-protein interactions (Østergaard and Yanofsky 2004). It is only by gathering all the information obtained in the different fields of functional genomics (*i.e.*, comparative genomics, transcriptomics, proteomics, metabolomics and phenomics) that plant researchers will be able to unequivocally assign a biological function to the unknown plant genes (Holtorf *et al.* 2002). For simplification, only some of the numerous analytical steps needed to initiate a biological validation are subsequently overviewed.

As pointed out in Chapter 3, functional genomics has been greatly based on reverse genetics approaches supported by an ever-increasing collection of *Arabidopsis* resources and tools. After selection of the gene or set of genes of particular interest, a combination of classical and recently available genome-wide approaches enables the testing and confirmation of hypotheses about specific gene functions (Alonso and Ecker 2006). *In silico* analysis based on sequence homology should be taken as an initial and simplest approach, even though several genes do not show sequence similarity to previously functional characterized genes (Feng and Mundy 2006). Nonetheless, relevant general information given by sequence homology may provide a clue to the potential cellular role of gene/protein. Bioinformatic studies provide indirect information on, for example, spatial and temporal expression patterns, subcellular localization and co-expression gene network, which can be functionally correlative as well (reviewed in Chapter 3). As *in silico* analysis is generally not sufficient to precisely define gene function, disruption of gene activity is currently the method of choice to experimentally validate a specific function (Feng and Mundy 2006).

Due to the extensive collection of insertion mutants available for ordering in stock centers, the role of a gene can be simply revealed by association with a detectable loss-of-function phenotype. Preceding the phenotypic analysis, diagnostic

primers (gene- and T-DNA-specific) are used in PCR amplification for identification of a homozygous line with an insertion in the gene of interest (Østergaard and Yanofsky 2004). If an insertion mutant is not available, an alternative could be the silencing of gene expression through a mechanism of RNA interference (RNAi). Whatever the method used, the successful observation of a mutant phenotype is not guaranteed and it depends on several factors, such as the existence of gene redundancy, the background of the mutant line and the occurrence of phenotype suppression or overlapping functionality between unrelated genes (reviewed by Østergaard and Yanofsky 2004). In addition, as reviewed in Chapter 4, some mutant phenotypes may only be detected when subjected to the appropriate assay conditions. Nevertheless, due to the high level of redundancy observed in *Arabidopsis*, only about 10% of genes are estimated to produce an observable loss-of-function phenotype. To circumvent this difficulty, double or triple knockout mutants can be constructed to uncover the hidden phenotypes.

Once a mutant phenotype is successfully detected, it is necessary to prove that it is indeed a consequence of the insertion of T-DNA into the gene of interest (Østergaard and Yanofsky 2004). Northern blot analysis or RT-PCR experiments can determine if the corresponding gene expression is definitely interrupted in the mutant plants. Also, the use of a T-DNA fragment as probe in a Southern blot analysis of digested genomic DNA ensures that only the desired insertion is present in the mutant, excluding the possibility that secondary mutations are responsible for the phenotype. Since second site mutations can spontaneously arise somewhere else in the genome, the use of additional mutant alleles or complementation studies is generally required for conclusive phenotype acceptance (Østergaard and Yanofsky 2004). Therefore, before assuming that the disrupted gene is undoubtedly responsible for the phenotype, a similar mutant phenotype should be detected in other insertion alleles or the mutant phenotype should be rescued through transformation with the wild-type gene.

The gene expression studies that identify the expression pattern of a gene are further approaches that provide additional supporting evidence for its functional role. Gene expression profiling of wild-type plants using Northern blot or RT-PCR analysis helps to elucidate in which process, stage of development or tissues the gene may

have a particular function. Determination of other functional aspects of the unknown gene/protein could be further achieved by the use of transgenes, the use of which has rapidly progressed by the recent development of a fast and highly efficient DNA cloning technology (Gateway® Technology, Invitrogen) and a set of vectors suitable for plant ectopic expression (Curtis and Grossniklaus 2003). *In situ* analyses using promoter-reporter (GUS) constructs and coding sequence-GFP fusions determines respectively the spatial/temporal expression pattern of the gene and the subcellular localization of the corresponding protein. Additionally, the effect of enhanced expression in gain-of-function mutants can be evaluated by placing the gene under the transcriptional control of a constitutive promoter.

Aiming to functionally characterize the heat-induced gene *HZF*, previously identified by analysis of publicly available transcriptome data (Chapter 3), some of the outlined steps were followed.

MATERIAL AND METHODS

In silico analysis

To gather structural and functional data available for *HZF* gene (At1g26800) and corresponding protein, a number of bioinformatics resources and databases were accessed (Table 5.1). Please refer to Chapter 3 for details.

Table 5.1 Bioinformatics resources and databases used for *HZF* gene/protein structural and functional analysis.

Resource/Database		Information
ARAMEMNON	*http://aramemnon.botanik.uni-koeln.de/*	Transmembrane domains Subcellular protein localization
ATTED-II	*http://www.atted.bio.titech.ac.jp/*	Co-expressed gene network
BAR	*http://www.bar.utoronto.ca/*	Subcellular protein localization
InterProScan	*http://www.ebi.ac.uk/Tools/InterProScan/*	Protein domains
NCBI Protein BLAST	*http://blast.ncbi.nlm.nih.gov/Blast.cgi*	Protein similarity
PredictNLS	*http://cubic.bioc.columbia.edu/predictNLS/*	Nuclear localization signal
SIGnAL T-DNA Express	*http://signal.salk.edu/cgi-bin/tdnaexpress*	T-DNA insertion mutants
TAIR	*http://www.arabidopsis.org*	Gene structure Protein domains and physical properties

Plant material and growth conditions

The *Arabidopsis thaliana* line (FLAG_311D05) containing a T-DNA insertion in the *HZF* gene (At1g26800) was obtained from the Versailles collection (Bechtold *et al.* 1993; Bouchez *et al.* 1993) and ordered via INRA resource center (http://dbsgap.versailles.inra.fr/publiclines). This insertion mutant line was established in *A. thaliana* Wassilewskija (Ws) background. Wild-type Ws ecotype seeds were ordered from the public stock center NASC (http://arabidopsis.info/; Scholl *et al.* 2000).

Seed stratification, sterilization and germination in sterile conditions, as well as planting on soil for bulk seed production, were performed as described in Chapter 4. For the successful recovery of seeds from individual plants, plant growth was performed using the ARASYSTEM (Lehle seeds) system.

Isolation of *hzf* homozygous insertion line

About 50 plants from the ordered mutant seeds stock were grown for diagnostic PCR and seed recovering. Genomic DNA from *Arabidopsis* leaves was isolated using the CTAB extraction method modified by Posé (2008) (Annex I). Screening for homozygous mutant lines was performed by diagnostic PCR analysis (Annex I), using one primer specific for the T-DNA insertion border (Lb4) and two primers conceived for the gene (HZF dpcr LP and HZF dpcr RP). *HZF* specific primers were designed using the SIGnAL T-DNA Primer Design tool (http://signal.salk.edu/tdnaprimers.2.html) and the primer analysis software Oligo6 (*Primer Analysis Software*, version 6.54). The design of gene-specific primers was performed taking into account a final length of approximately 20 nucleotides and a closely match on priming efficiency and melting temperatures values. Primer sequences and PCR conditions used for genotyping are presented in Annexes II and III. After gel electrophoresis analysis (Annex I), F1 homozygous seeds from each plant displaying the proper estimated fragments size (*hzf* mutants) were collected in individual containers. The analysis was repeated for F1 seedlings and for the next two generations to guarantee that the T-DNA insertion remained stable.

Thermotolerance assays

Arabidopsis wild-type (Ws) and *hzf* plants were grown under the same conditions for synchronized seed production for performing thermotolerance assays. Basal thermotolerance was evaluated in wild-type and mutant seeds using the germination assays described in Chapter 4. Immediately after removal from 4°C, stratified and sterilized seeds were heat-stressed at 50°C for different periods (15-300 min) or heat-treated for 60 min at increasing temperatures (38-56°C). After treatment, the seeds were plated onto MS medium and allowed to grow in standard conditions (23°C). After 2 days, the number of seeds showing radicle emergence was counted every day over a total period of 11 days. Results are presented as percentage of untreated germinated seeds. Mean and SEM were derived from four independent replicates, all containing 30 seeds of each condition tested.

Basal and acquired thermotolerance of 7-day-old seedlings of wild-type and *hzf* plants were assessed using the seedling survival assays described in Chapter 4. Briefly,

for basal thermotolerance evaluation, plates containing 7-day-old seedlings were submersed at 45°C for different periods (5-45 min) or at distinct temperatures (40-50°C) for 20 min. For acquired thermotolerance evaluation, 7-day-old seedlings were pre-incubated at different acclimation temperatures (30-40°C) for 60 min, followed by recovery at 23°C for 120 min and subsequent heat challenge at 45°C for 20 min. The survival rates were determined after 6 days of seedling growth. Mean and SEM were derived from three independent replicates for each condition tested, all containing 50 seedlings of both genotypes tested.

Gene expression analysis by RT-PCR amplification

To determine the *HZF* expression during heat treatment, wild-type and *hzf* seedlings grown for 16 days on MS agar plates were subjected to a heat stress of 38°C for 60 min. Samples were taken immediately before heat treatment (0 min), at 15, 30, and 60 min during heat stress. Four to five seedlings of each line were randomly selected and frozen in liquid nitrogen.

Total RNA was isolated using the TRIzol method (Annex I), originally described by Chomczynski and Sacchi (1987). After evaluation of RNA concentration and purity (Annex I), RNA was used as template for first-strand cDNA synthesis using Superscript® VILO™ cDNA Synthesis Kit (Invitrogen), according to suppliers instructions. Briefly, 1 µg of total RNA was gently mixed with 4 µl of 5x VILO™ Reaction Mix (Invitrogen), 2 µl of 10x SuperScript® Enzyme Mix (Invitrogen) and RNase-free water for a final volume of 20 µl. Following a first incubation at 25°C for 10 min, the reaction was further incubated at 42°C for 60 min. Finally, the reverse transcriptase was inactivated by heating at 85°C for 5 min. The cDNA was then used for PCR amplifications previously optimized for the number of cycles that fit into the linear amplification zone. PCR reactions were prepared as described in Annex I and amplifications were performed using gene-specific primers (HZF RT F and HZF RT R; Annex II) and 37 cycles (Annex III). As positive control, a RT-PCR reaction was performed in the same conditions using *ACTIN 2* (At3g18780) specific primers (ACT2 RT F and ACT2 RT R; Annex II) and 31 cycles (Annex III). The PCR products were analyzed by agarose gel electrophoresis (Annex I).

Construction of transformation vectors

Construction of transformation vectors was accomplished using the Gateway® recombination cloning system (Invitrogen), according to the instruction manual. *HZF promoter::GUS* constructs were generated by inserting the 482 bp predicted promoter region in the pMDC164 vector. For accomplishing this, the promoter sequence had been predicted using AGRIS resource (http://arabidopsis.med.ohio-state.edu/) and comprised the sequence just upstream of the start codon of *HZF*. *35S::HZF ORF* fusions were prepared by inserting the 615 bp *HZF* coding region in the pMDC32 vector under the control of the dual *35S* promoter. *HZF ORF-stop::GFP6his* constructs resulted from the insertion of the coding region of *HZF* without the stop codon in the pMDC85 vector, which also harbors the dual *35S* promoter. All destination vectors are represented in Annex IV and were ordered from ABRC (http://www.arabidopsis.org).

The three fragments of interest of *HZF* gene (promoter region, ORF and ORF without the stop codon) were amplified and flanked by *att*B regions by two-rounds of PCR amplifications using wild-type (Ws) genomic DNA (Annex I). The *HZF* specific primers were designed using the primer analysis software Oligo6 (*Primer Analysis Software*, version 6.54) in order to have 22-26 nucleotides and a closely match on priming efficiency and melting temperatures values. The first PCR was performed with sequence-specific primers (Annex II) containing part of the *att*B1 or *att*B2 recombination sites (12 bp) coupled at each 5'end. A second PCR was performed with adapter primers (*att*B1 and *att*B2; Annex II) for amplification of the fragment of interest containing the full sequence of *att*B regions. Primer sequences and PCR conditions are presented in Annexes II and III. The *att*B-PCR products were subsequently purified by PEG precipitation (Annex I) and introduced into the donor vector pDONR^{TM}201 (Invitrogen; Annex IV) by BP recombination reaction (Annex I). Aliquots of 5 µl of the BP reaction were used to transform *Escherichia coli* XL1-Blue competent cells by *SEM* (Annex I) and transformants were selected in LB medium supplemented with 50 µg.ml^{-1} kanamycin. The transformation efficiency was evaluated by colony PCR using the primers pDONR201 Fo and pDONR201 Rv (Annex II). The PCR products were separated on agarose gel electrophoresis and positive transformants clones were selected according to the expected fragments size. The cloned sequences

were then exported to the suitable destination vector (Curtis and Grossniklaus 2003) through the LR reaction (Annex I). As performed after BP reaction, *E. coli* cells were transformed with the generated vectors and positive clones were identified by colony PCR. The new constructs were subsequently used to transform *Agrobacterium tumefaciens* cells aimed for *A. thaliana* transformation. After selection in LB medium supplemented with 50 $\mu g.ml^{-1}$ kanamycin and 50 $\mu g.ml^{-1}$ rifampycin, five clones were verified by colony PCR using the same specific primers.

Generation of transgenic plants

All the *HZF*-based constructs produced using the Gateway® cloning technology were used to transform wild-type plants. Transformation of *Arabidopsis* was performed by the floral dip method (Clough and Bent 1998) using *A. tumefaciens* strain EHA105 (Annex I). For the successful recovery of seeds from transformed plants (T0), plant growth was performed as described previously. *Arabidopsis* T1 plants were germinated on MS plates containing 50 $\mu g.ml^{-1}$ hygromycin and the resistant plants were transferred to soil to obtain T2 seeds. The surviving ability of tested T2 lines on hygromycin MS plates would allow determining if the transgene is segregating in a Mendelian fashion. If this is the case, a single insertion of transgene should have occurred. A second round of selection was performed from each individual plant, in order to determine the genotype of T3 plants and use them for further experiments.

RESULTS AND DISCUSSION

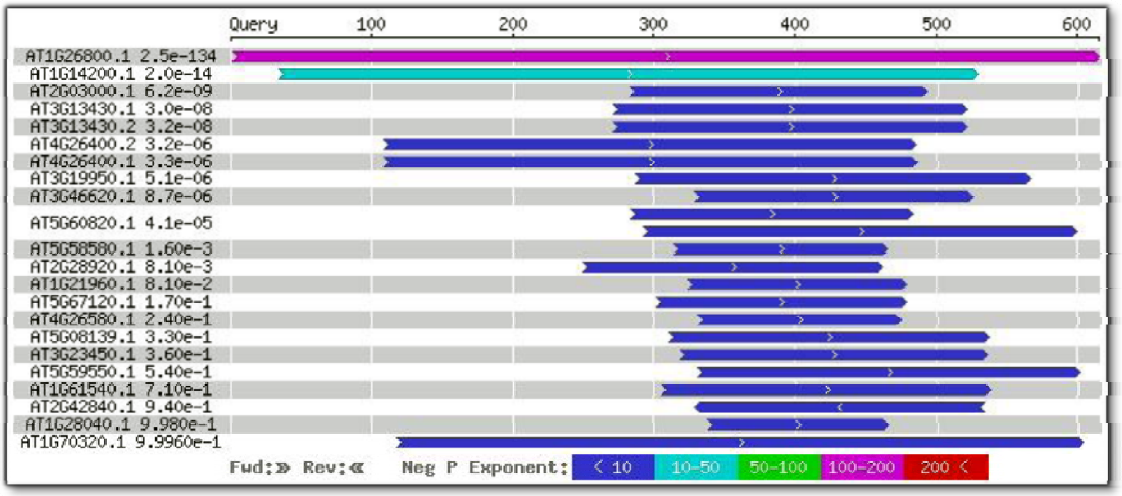
In silico analysis of HZF: gene and protein structural information

Relevant information on *HZF* (At1g26800) gene structure and gene product was obtained in TAIR database. Due to the presence of an InterPro zinc finger domain, the corresponding gene product has been annotated as a zinc finger (C3HC4-type RING finger) family protein. HZF is encoded on reverse strand of chromosome 1 of *A. thaliana* from 9286311 to 9285086 bp (Figure 5.1). The *HZF* full length genomic sequence covers 1226 bp with no introns and the resultant coding sequence (CDS) comprises 615 bp.

Figure 5.1. Genomic DNA sequence of *HZF* (At1g26800) on *Arabidopsis* chromosome 1. Coding sequence nucleotides (CDS) are located from 9286177 to 9285563 bp (in orange caps). Start and termination translational codons are illustrated by blue boxes and the 3' and 5' UTR regions are depicted in red letters. Image was obtained using TAIR SeqViewer.

Using the TAIR external link WU-BLAST, the *HZF* CDS and full length genomic sequence were aligned with similar *Arabidopsis* sequences. The coding sequence was searched against *TAIR8 CDS (-introns, +UTRs)* database and the highest significant alignments were observed with several sequences of other uncharacterized zinc finger (C3HC4-type RING finger) family proteins (Figure 5.2A). The sequence identities ranged between 55 and 65% (Table 5.2). When full length genomic sequence was queried against *TAIR8 Genes (+introns, +UTRs)* database, in addition to the same zinc finger family proteins, two different myb family transcription factors showed significant alignments (Figure 5.2B). Furthermore, these myb-coding sequences aligned antisense to the 3' UTR region of *HZF*. Considering the high sequence identities observed for both myb family transcription factors (70% for At1g69580 and 65% for At5g29000,

A



B

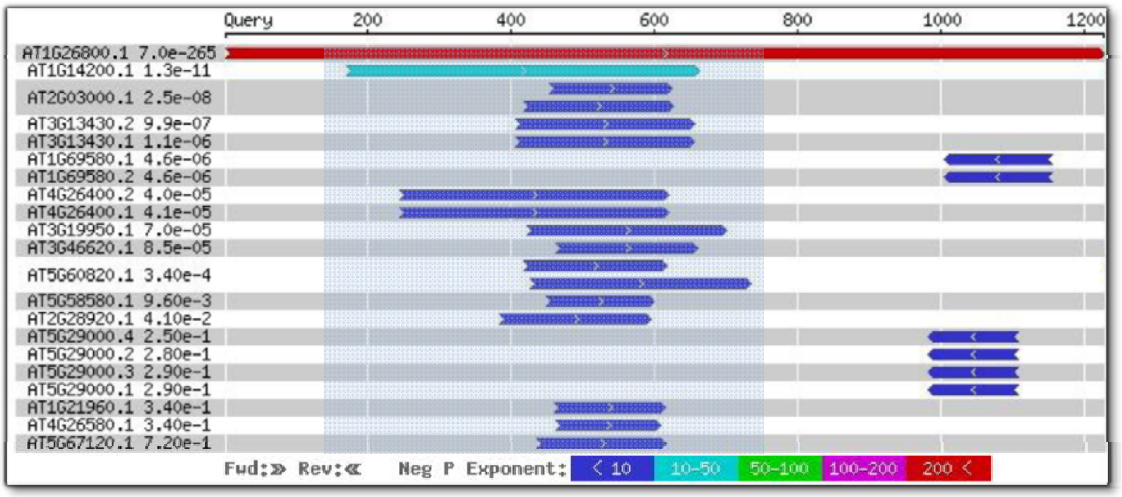


Figure 5.2. Graphic representation of the 20 highest *Arabidopsis* sequences producing significant alignment with *HZF* sequences. The coding sequence was compared against *TAIR8 CDS (-introns, +UTRs)* database (A) or the full length genomic sequence was searched against *TAIR8 Genes (+introns, +UTRs)* database (B). Locus description and identities are presented in Table 5.2. Analysis was performed using TAIR external link WU-BLAST. In Figure B the coding region is highlighted by a blue box.

Table 5.2), it would be possible to hypothesize a putative interaction of myb-coding sequences with *HZF*, integrated in a post-transcriptional regulator mechanism. Among the other uncharacterized zinc finger (C3HC4-type RING finger) family proteins producing significant alignment with both *HZF* CDS and full length genomic sequence, At1g14200 gene (further referred to as *L-HZF*) displayed the most similar sequence. This high degree of sequence similarity may suggest the possible occurrence of overlapping gene functionality.

TAIR predictions on protein domains and physical properties, such as molecular

Table 5.2 Locus description of *Arabidopsis* sequences presenting the highest alignment with *HZF* and corresponding identities to *HZF*. BLAST was performed as described in Figure 5.2. Sequences are ordered according to CDS BLAST score results.

Locus	Description	Identities (CDS BLAST)	Identities (genomic BLAST)
AT1G14200.1	zinc finger (C3HC4-type RING finger) family protein		59%
AT2G03000.1	zinc finger (C3HC4-type RING finger) family protein		65%
AT3G13430.1	zinc finger (C3HC4-type RING finger) family protein		62%
AT3G13430.2	zinc finger (C3HC4-type RING finger) family protein		62%
AT4G26400.2	zinc finger (C3HC4-type RING finger) family protein		57%
AT4G26400.1	zinc finger (C3HC4-type RING finger) family protein		57%
AT3G19950.1	zinc finger (C3HC4-type RING finger) family protein		58%
AT3G46620.1	zinc finger (C3HC4-type RING finger) family protein		64%
AT5G60820.1	zinc finger (C3HC4-type RING finger) family protein		63%
AT5G58580.1	ATL63; protein binding / zinc ion binding		65%
AT2G28920.1	zinc finger (C3HC4-type RING finger) family protein		60%
AT1G21960.1	zinc finger (C3HC4-type RING finger) family protein		62%
AT5G67120.1	zinc finger (C3HC4-type RING finger) family protein		61%
AT4G26580.1	protein binding / zinc ion binding		63%
AT5G08139.1	zinc finger (C3HC4-type RING finger) family protein	58%	-
AT3G23450.1	pseudogene	60%	-
AT5G59550.1	zinc finger (C3HC4-type RING finger) family protein	57%	-
AT1G61540.1	kelch repeat-containing F-box family protein	58%	-
AT2G42840.1	PDF1 (PROTODERMAL FACTOR 1)	60%	-
AT1G28040.1	protein binding / zinc ion binding	63%	-
AT1G70320.1	UPL2 (UBIQUITIN-PROTEIN LIGASE 2)	55%	-
AT1G69580.1	transcription factor	-	70%
AT1G69580.2	similar to myb family transcription factor	-	70%
AT5G29000.4	myb family transcription factor	-	65%
AT5G29000.2	myb family transcription factor	-	65%
AT5G29000.3	myb family transcription factor	-	65%
AT5G29000.1	myb family transcription factor	-	65%

weight and isoelectric point, were obtained from a variety of sources including TIGR (*The Institute for Genomic Research*) and InterPro databases (Garcia-Hernandez *et al.* 2002). HZF has a protein length of 204 aa (Figure 5.3) with a predicted molecular weight of 22.74 kDa and an isoelectric point of 4.29. Prediction of protein domains using InterProScan identified a conserved zinc finger RING-type domain located between amino acid residues 113 and 153 of HZF sequence (Figure 5.3). Although in

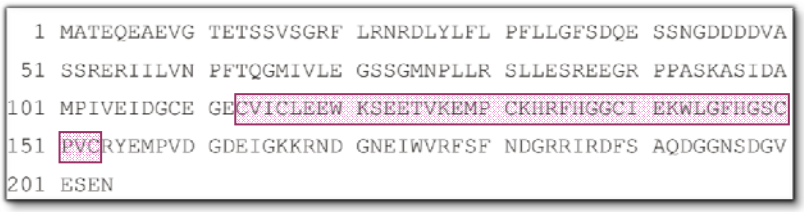


Figure 5.3. Amino acidic sequence of HZF. Amino acids are shown using the one-letter code and numbers on the left refer to amino acids numbers. Colored box indicates the conserved C3H2C3-type RING finger domain located from aa 113 to 153.

TAIR resource this gene has been annotated as a zinc finger family protein containing a C3HC4-type RING finger domain, the analysis of the previously defined conserved domain allowed the recognition of a C3H2C3-type sequence motif. The C3H2C3-type domain of HZF comprise a sequence of 41 amino acid residues, namely C-X2-C-X14-C-X1-H-X2-H-X2-C-X10-C-X2-C, in which X is any amino acid and four pairs of ligands putatively bind two zinc ions forming a "cross-brace" motif (Borden and Freemont 1996; Saurin *et al.* 1996). The RING (*Really Interesting New Gene*) finger motif, which comprises C3HC4- or C3H2C3-type variants has been shown to function as a protein-protein interacting domain (Freemont *et al.* 1991; Saurin *et al.* 1996).

A C3H2C3-type RING finger domain was already reported for HZF protein when manual curation and computational analysis were employed for grouping 387 *Arabidopsis* RING domains according to their metal-ligand arrangement (Kosarev *et al.* 2002). Also in this study, all of the previously described zinc finger (C3HC4-type RING finger) family proteins that showed significant alignment with *HZF* sequence (Table 5.2) were classified as C3H2C3-type RING finger-containing proteins, with the exception of At1g21960.

Biochemical and genetic evidences for ubiquitin ligase activity of RING finger domain proteins have been reported (reviewed by Jackson *et al.* 2000; Joazeiro and Weissman 2000). In a more recent study, a significant number of the 469 manually curated RING domain-containing proteins in *Arabidopsis* were demonstrated to function as E3 ubiquitin ligases by interaction with E2 conjugating enzymes (Stone *et al.* 2005). For establishing this interaction with E2 enzymes, the RING domain was found to be essential. The ubiquitin-proteasome pathway promotes the targeting of proteins for degradation in a three-step reaction cascade catalyzed by E1, E2 and E3 enzymes (reviewed by Smalle and Vierstra 2004). In the final step, E3 ligases bind

directly to the target protein, mediating the transfer of ubiquitin from an interacting E2 enzyme to a lysine residue in the substrate. Therefore, E3 ligases are responsible for providing reaction specificity to ubiquitination, which is reflected in the high number of potential E3 ligases encoded by the *Arabidopsis* genome (reviewed by Stone *et al.* 2005). RING-type E3 ligases and U-box containing enzymes are included in one of the four described classes of ubiquitin ligases, which also comprise HECT-, SCF-, and APC-type enzymes (reviewed by Smalle and Vierstra 2004). Most of the RING proteins displays E3 ligase activity and their implication in the regulation of different cellular processes, like hormone regulation, embryogenesis, senescence, pathogen defense, flower development, photomorphogenesis and ABA signaling has been reported (reviewed by Sullivan *et al.* 2003; Moon *et al.* 2004; Bu *et al.* 2009). Recently, an important role for RING finger proteins in degrading regulator proteins has been suggested in response to abiotic stress, specifically in cold (Dong *et al.* 2006), salt and drought responses (Ko *et al.* 2006; Zhang *et al.* 2007), and temperature tolerance (Yan *et al.* 2003).

The TAIR external link WU-BLAST was used to find similar *Arabidopsis* proteins. HZF amino acidic sequence was compared against *TAIR8 Proteins* database and similar proteins aligned, mainly because of the conserved domain region localized at the C-terminus (data not shown). The most significant alignments were attained for functionally uncharacterized zinc finger family proteins. An exception was found for CIP8 (COP1-interacting protein 8; At5g64920), a zinc finger family protein containing a C3H2C3-type RING motif displaying ubiquitin ligase activity (Hardtke *et al.* 2002). CIP8 protein was suggested to form a minimal ubiquitin ligase that interacts with COP1. Through the cooperation with an E2 enzyme, this interaction results in the ubiquitination of the transcription factor HY5, a positive regulator of photomorphogenesis. Interestingly, HZF similarity to the ubiquitin ligase CIP8 has been previously reported in the analysis of *Arabidopsis* RING domains performed by Kosarev *et al.* (2002). In this study, it was established that the proteins included in the same cluster as HZF, which comprised 26 proteins (including CIP8), display significant similarity outside the RING domain. It was also suggested that a subgroup of this cluster presenting high structural similarity with CIP8 (and this fact was verified for HZF), might have ubiquitin ligase activity as demonstrated for CIP8 (Hardtke *et al.*

2002). Furthermore, this cluster was suggested to include proteins that might be functionally redundant.

Altogether, the existence of a RING finger domain in HZF and protein alignment results (namely the similarity with CIP8) suggest that HZF may be involved in the regulation of protein levels under heat stress conditions, possibly functioning as an E3 ubiquitin ligase. Also, the protein to which HZF displayed the highest degree of similarity among the uncharacterized zinc finger family proteins (L-HZF) is included in the same cluster of HZF and CIP8 in the study performed by Kosarev *et al.* (2002). According to the authors, HZF could have a redundant function with L-HZF.

Proteins similar to HZF in other organisms were searched across *non-redundant protein sequences* database using NCBI BLASTP program (http://www.ncbi.nlm.nih.gov/; Altschul *et al.* 1997). The closest relatives to HZF were found in *Populus trichocarpa*, *Vitis vinifera* and *Populus trichocarpa* x *Populus deltoides* species, with identities values ranging between 54% and 60% (data not shown). The amino acidic sequences producing significant alignment are presented according to the *lineage report* provided by BLASTP results (Figure 5.4). In this report, similar proteins are displayed by organisms, grouped according to their taxonomic classification. A great number of proteins similar to HZF were found for most of the plant species with completed genome sequencing. This large number of potential E3 ubiquitinating enzymes suggests once more that substrate-specific ubiquitination performed by

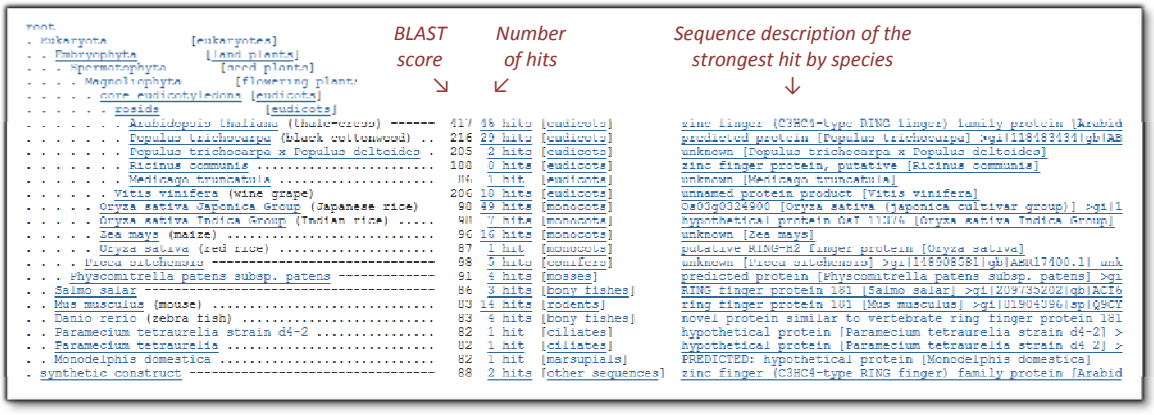


Figure 5.4. Lineage report of the proteins producing the most significant alignment with HZF. The amino acidic sequence was searched against *non-redundant protein sequences* database. Protein similarity between different organisms is viewed according to their classification in the *taxonomy* database. The number of hits by species is presented in the center as blue numbers and BLAST score for the strongest hit for each species is depicted in black numbers. Analysis was performed using NCBI BLASTP program (Altschul *et al.* 1997).

RING-type E3 ligases is an essential mechanism of cellular regulation in the majority of plants.

According to ARAMEMNON prediction, HZF is probably a soluble protein. No transmembrane spans were detected and subcellular location was weakly predicted for chloroplast, mitochondrion or secretory pathways. As shown in Chapter 3, strong prediction for nuclear localization was found in Cell eFP Browser tool provided by BAR, although no nuclear localization signal (NLS) was detected in PredictNLS server (Cokol *et al.* 2000).

In silico analysis of HZF: expression information

As referred in Chapter 3, co-expressed gene relationships predicted in ATTED-II database can assist in the functional characterization of the gene. Some of the most co-regulated genes for *HZF*, presented in Table 3.5 (Chapter 3), can be visualized integrated in a more complex gene network (Figure 5.5). In this analysis, *HZF* is

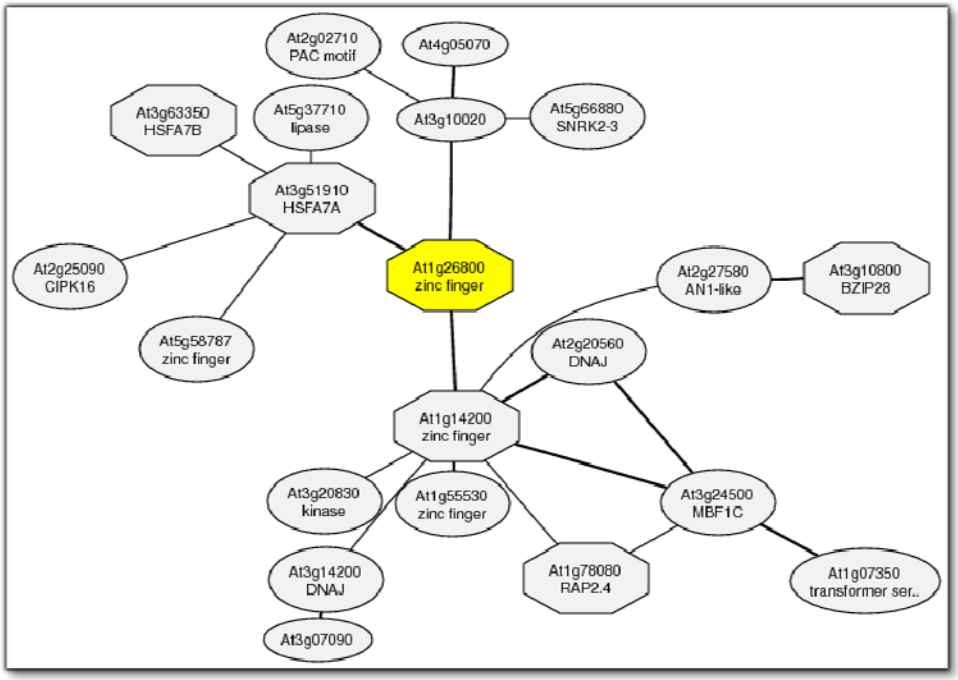


Figure 5.5. Graphic representation of *HZF* co-expression gene network drawn based on rank of correlation. The gene of interest is highlighted in yellow and stronger relationships are represented by bold lines. Genes represented by octagon-shape nodes are predicted transcription factors whereas other genes are symbolized by circle-shape nodes. Whenever a function is known/predicted for a gene, an indication is displayed below the corresponding AGI number. Locus description of some of the *HZF* most co-regulated genes integrated in this network is presented in Table 3.5 (Chapter 3). Analysis was performed in ATTED-II database.

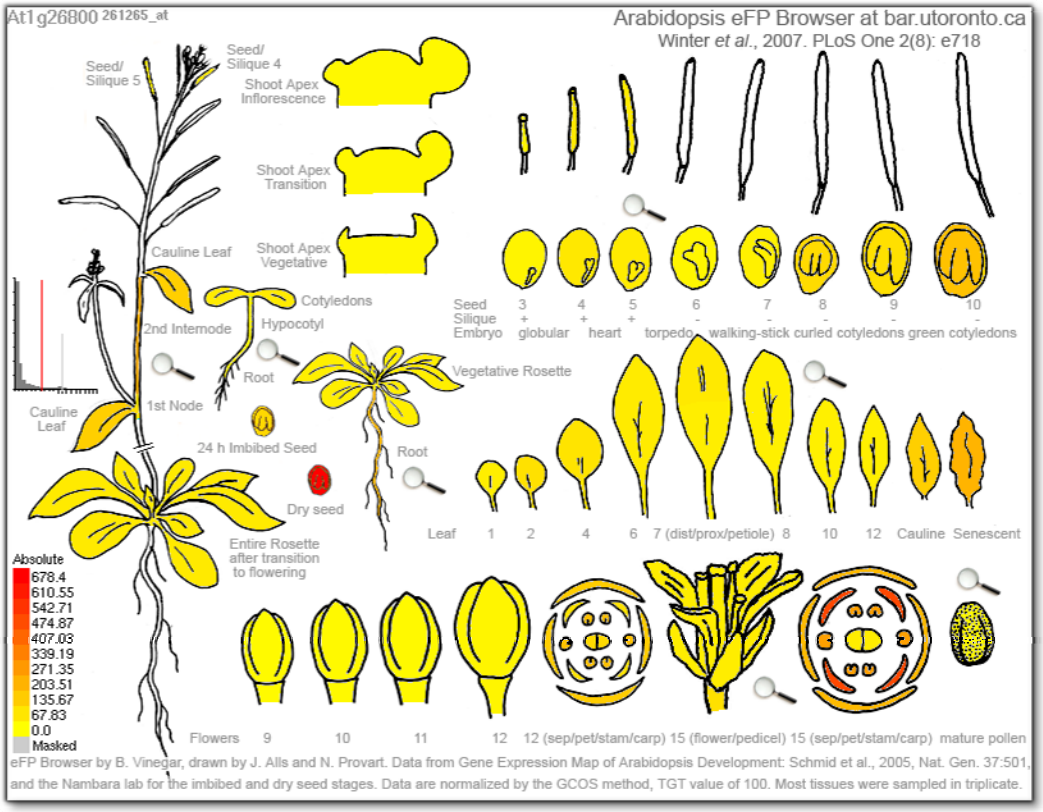
suggested to be involved in the initiation of heat stress response, since it presents high co-expression with *HSF* genes. Due to its RING-finger domain that is probably involved in the ubiquitination of specific proteins, HZF might be related with the degradation of certain specific regulator proteins that control the expression of other genes. The involvement of *HZF* in regulatory mechanisms can be presumed due to the strong co-expression with the transcription factor *HSFA7a* (At3g51910), which is in turn highly correlated with another *HSF* (*HSFA7b*, At3g63350). Additionally, *HSFA2*, *HSFB2a* and *HSFB2b* expression is, to a certain extent, directly connected with *HZF* expression (data not shown). The analysis of the transcript profiles of HSF-encoding genes during abiotic stress responses allowed to determine which *HSF* transcripts are most strongly induced by heat stress (Von Koskull-Döring *et al.* 2007). Those transcripts are *HSFA2*, *HSFA7a*, *HSFA7b*, *HSFB1*, *HSFB2a* and *HSFB2*. Interestingly, nearly all appeared to be co-expressed with *HZF*, suggesting a putative role of HZF in heat response.

The *HZF* gene expression during *Arabidopsis* life cycle can be predicted using Arabidopsis eFP Browser (Winter *et al.* 2007), available in BAR, by creating an electronic fluorescent pictographic representation. During development stages of wild-type *Arabidopsis*, *HZF* gene displays a strong expression level in dry seeds and petals of mature flowers (Figure 5.6A). During the first hour of seed imbibition, the expression levels are still relevant but strongly decreased for longer imbibition periods (Figure 5.6B). The *HZF* transcription levels are also evident in green cotyledons, stems, senescing leaves, sepals and stamens of mature flowers and in the roots of the vegetative rosette (Figure 5.6A).

Isolation of *hzf* homozygous insertion line

With the recent increase in insertion mutant availability and gene silencing strategies, functional gene discovery by mutant phenotype association became a simpler strategy. This task has been facilitated by the easily ordering of mutant seeds from stock centers collections (Chapter 3). Selection of the appropriate T-DNA insertion mutant for *HZF* gene was performed in TAIR database, together with SIGnAL T-DNA Express tool. Several insertion lines were found to interrupt *HZF* gene. Preference was given to the interruption in the exons that would result in loss-of-

A



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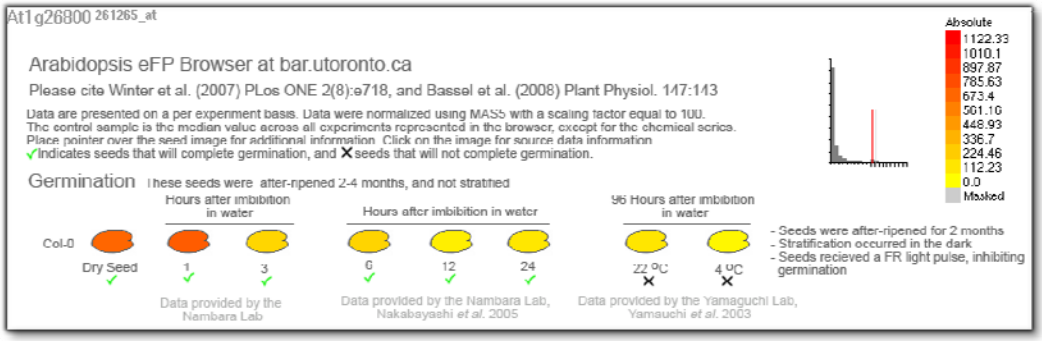


Figure 5.6. Gene expression pattern of *HZF* gene as an "electronic fluorescent pictographic" representation, during development of *A. thaliana* wild-type tissues (A) and seed germination (B). Expression levels are identified in colors as depicted in the bars. This analysis was performed using Arabidopsis eFP Browser provided by BAR, considering the Development Map (A) and Seed (B) data sources in the absolute mode.

function alleles. A FLAG T-DNA line (FLAG_311D05) in Ws ecotype, with an insertion located in 9285899 bp of the single exon of *HZF* (refer to Figure 5.1), was ordered (Figure 5.7).

As the plant material is received in the form of segregating flank-tagged T3-generation seeds, a diagnostic PCR (using two gene-specific and one insertion-specific primers) was performed for the determination of homozygous insertion T-DNA lines. A

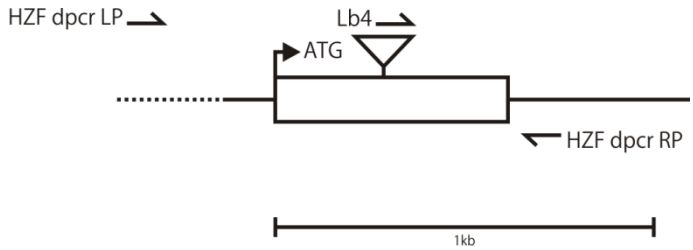


Figure 5.7. Schematic representation of the *A. thaliana HZF* T-DNA inserted gene. The T-DNA insertion (triangle) is localized in the exon region of the gene (white box). The predicted promoter region is depicted by a dashed line and the full line indicates the 5' and 3' UTR. The arrows represent the diagnostic primers used for genotyping by PCR analysis.

simplified *HZF* gene scheme is represented in Figure 5.7, with particular emphasis on T-DNA insertion localization and diagnostic primers used. According to the protocol provided by SIGnAL T-DNA Primer Design, the number and dimension of the amplified fragments size reveals the plant genotype. Using the three referred primers (Lb4 + HZF dpcr LP + HZF dpcr RP; Annex II), wild-type plants (no insertion) should amplify a PCR product of about 1141 bp (from HZF dpcr LP to HZF dpcr RP), homozygous lines (insertions in both chromosomes) will get a predicted band of 582-882 bp (from HZF dpcr RP to Lb4), and heterozygous lines (one insertion in a single chromosome) will get both bands. After genomic DNA purification from individual plants and diagnostic PCR, amplification products were observed by gel electrophoresis analysis and three homozygous T-DNA insertion lines were obtained (Figure 5.8).

With the isolation of *hzf* homozygous lines, tools for pursuing a reverse genetics approach through association of heat phenotype to this potential thermotolerance determinant were developed. The stability of the insertion was confirmed for the next two generations of homozygous mutant plants and seed progeny was preserved for further functional analysis.

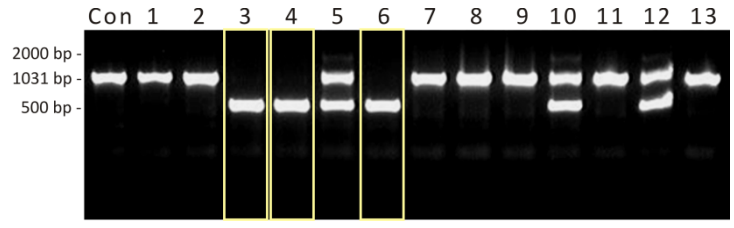


Figure 5.8. Electrophoretic analysis of diagnostic PCR products for genotypic identification of homozygous *HZF* gene knockout lines. Publicly available *HZF* T-DNA insertion line was ordered and each segregating seed was tested for homozygous T-DNA inserts by diagnostic PCR. Genomic DNA of each tested plant and primers illustrated in Figure 5.7 (Annex II) were used for diagnostic PCR. The identified mutant lines are evidenced by yellow boxes. Control (Con) was performed using a wild-type *Arabidopsis* Ws plant.

Phenotypic analysis of loss-of-function *hzf* mutants

As previously discussed in Chapter 4, searching for a phenotype is a quite challenging and often laborious task. By using the thermotolerance assays previously developed for an easy detection of phenotypic alterations (Chapter 4), the evaluation of heat sensitivity of *hzf* was possible. The mutant line was tested for basal thermotolerance at seed level with variable times (15-300 min) of heat treatment at 50°C, as well as variable temperatures (38-56°C) of 60 min treatment. The effect of heat treatment on *hzf* seeds was evaluated by measuring the total number of germinated seeds along 11 days following heat stress.

The results presented for both assays were obtained 4 days after treatment (Figure 5.9A and 5.10A). When compared to wild-type *Arabidopsis* Ws, *hzf* seeds appeared to be more defective in germination after incubation at 50°C for periods of 15 and 30 min (Figure 5.9A). This germination defect became less severe with increasing germination periods (Figure 5.9B). When considering 11 days after stress treatment, a similar percentage of germinated wild-type and *hzf* mutant seeds was

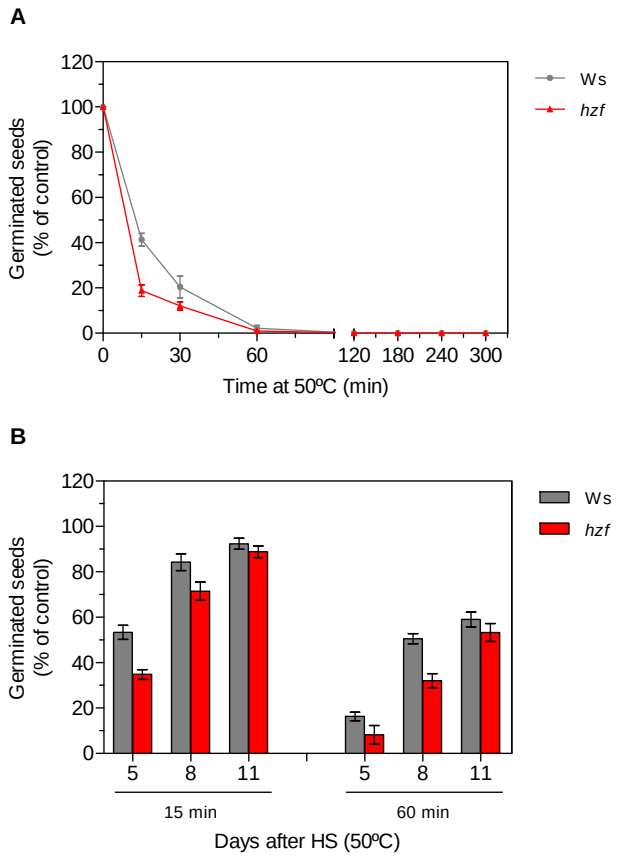


Figure 5.9. Assessment of basal thermotolerance in *hzf* mutant seeds using a germination assay with increasing times of treatment. After stratification at 4°C, seeds were treated at 50°C for different periods (15-300 min) and subsequently plated in MS agar. The number of germinated seeds was measured every day following a period of 2 days after treatment until 11 days upon heat stress imposition. Percentage of germination, in relation to untreated seed germination, was determined after 4 days of heat stress imposition and plotted as a function of heat stress treatment period (A). Germination percentages, in relation to untreated seed germination, were also determined for 5, 8 and 11 days after heat stress during 15 and 60 min (B). Wild-type *Arabidopsis* Ws seeds were included in all plates and subjected to identical assay conditions. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

detected for all the stress treatments (data not shown). An identical effect on germination was noticed when increasing the temperatures for a 60 min treatment (Figure 5.10A). Treatment at 47 or 50°C resulted in considerable differences in the number of *hzf* and Ws germinated seeds 4 days after stress (Figure 5.10B). Once again, this effect was restrained when considering longer germination periods. However, in contrast to the previous assay, identical levels of germination to wild-type seeds were never achieved, at least up to 11 days after heat treatment (Figure 5.10B).

In both germination assays, a phenotypic difference on germination ability between *hzf* and wild-type seeds was detected upon heat treatment. The germination of mutant seeds suffered a delay during the first days upon heat stress, in comparison with the wild-type control, but soon recovered and achieved similar germination rates to wild-type. According to Bentsink and Koornneef (2008), mutants displaying altered seed germination can be affected in genes implicated on germination or dormancy. Accordingly, a better or faster mutant germination indicates the involvement of the disrupted gene in promoting dormancy or repressing germination. In this work, the

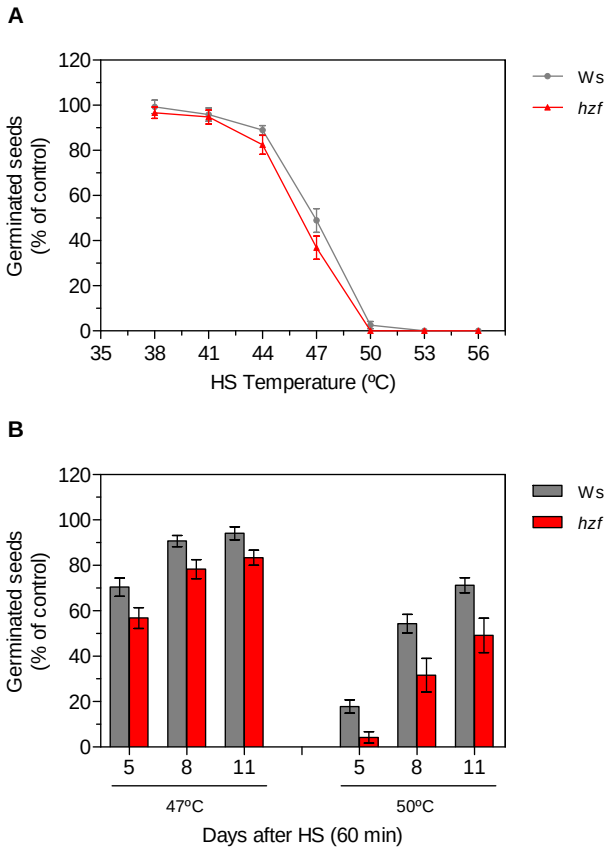


Figure 5.10. Assessment of basal thermotolerance in *hzf* mutant seeds using a germination assay with increasing temperatures of treatment. After stratification at 4°C, seeds were treated at different temperatures (38-56°C) for 60 min and subsequently plated in MS agar. The number of germinated seeds was measured every day following a period of 2 days after treatment until 11 days upon heat stress imposition. Percentage of germination, in relation to untreated seed germination, was determined after 4 days of heat stress imposition and plotted as a function of heat stress treatment temperature (A). Germination percentages, in relation to untreated seed germination, were also determined for 5, 8 and 11 days after heat stress at 47 and 50°C (B). Wild-type *Arabidopsis* Ws seeds were included in all plates and subjected to identical assay conditions. Mean and SEM were derived from measurements in four replicates for each genotype, all containing 30 seeds of each condition tested.

results suggest that *HZF* may have a particular role in the transition from the dormant to the germinating state under heat stress, facilitating the germination process. Concerning the suggested function of HZF as an E3 ubiquitin ligase and the role of these proteins in target-specific proteolysis, it is possible that HZF may control the levels of a regulator protein that induces seed dormancy or inhibits germination. It is important to note that under standard conditions *hzf* and wild-type mutant seeds display similar germinations rates, suggesting that the role of HZF will be only essential for germination after challenging temperatures. After four days of germination without previous heat stress imposition on seeds, the germination rate of *hzf* seeds was ~91% against ~90% in wild-type seeds.

Basal thermotolerance was further evaluated in 7-day-old *hzf* seedlings by challenging them at 45°C for 5-45 min (Figure 5.11A) or at 40-50°C for 20 min (Figure 5.11B). The mutant seedlings were also tested for acquired thermotolerance by acclimation at 30-40°C for 60 min, followed by recovering at 23°C for 120 min and heat treatment at 45°C for 20 min (Figure 5.11C). The survival rate was always determined 6 days after treatment. For all the conditions imposed, no significant differences were detected in seedling survival between the wild-type and mutant plants, suggesting that *HZF* may be not essential for protecting *Arabidopsis* seedlings from heat stress. However, the possibility of gene redundancy cannot be excluded. Indeed, a high degree of functional redundancy among *Arabidopsis* RING-domain proteins had already been suggested (Kosarev *et al.* 2002).

Southern blot analysis for determination of the number of T-DNA insertions is still under progress, as well as gene complementation studies and gain-of-function mutant phenotype analysis. It would be also important to confirm the observed mutant phenotype by using other mutant *HZF* alleles in the same phenotypic analysis. Additional *HZF* insertion alleles are currently available in stock centers, either with a T-DNA insertion in the promoter (*e.g.*, SALK_002287 or WiscDsLox383F11) or in the exon (*e.g.*, ossowski_1132128 or FLAG_324E09). Altogether, these assays will allow a conclusive validation of the heat-associated phenotype observed at seed stage.

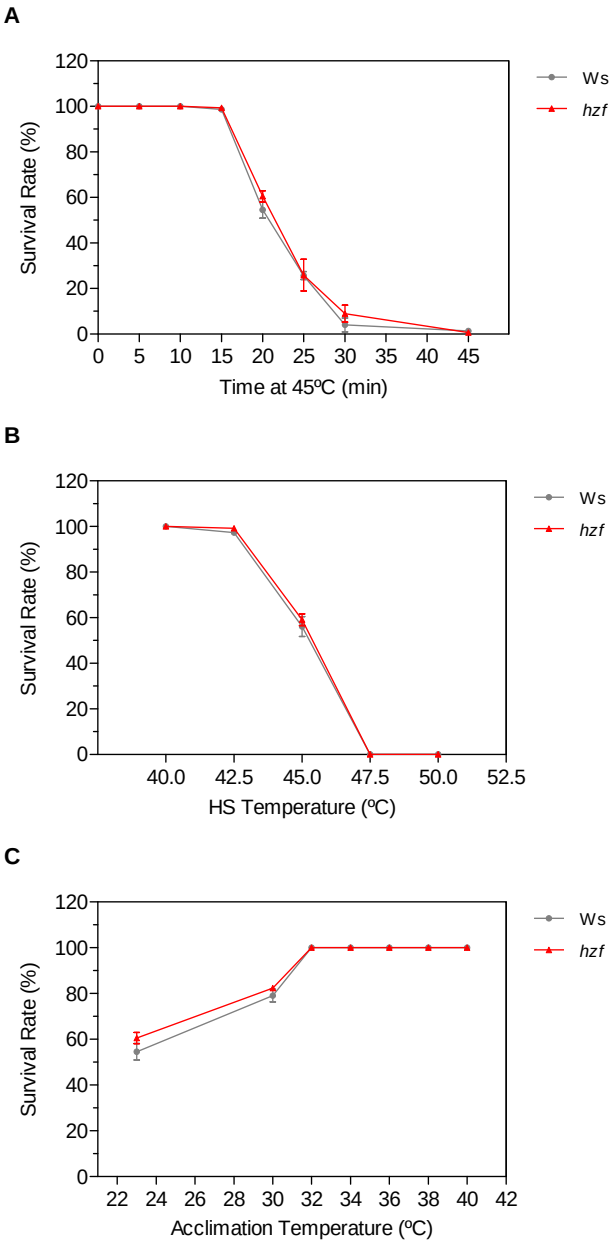


Figure 5.11. Assessment of basal and acquired thermotolerance in *hzf* mutant seedlings using survival assays. Seven-day-old seedlings grown in MS agar plates were: (A) treated at 45°C for different periods (5-45 min), followed by recovery at 23°C; (B) treated at different temperatures (40-50°C) for 20 min, followed by recovery at 23°C, or (C) pre-conditioned at different temperatures (30-40°C) for 60 min, recovered at 23°C for 120 min and then further treated at 45°C for 20 min, followed by recovery at 23°C. The number of viable seedlings was counted 6 days after heat stress and plotted as a percentage of survival. Wild-type *Arabidopsis* Ws seedlings were included in all assays and subjected to identical assay conditions. Mean and SEM were derived from measurements in three replicates in the same conditions, all containing 50 seeds of each genotype tested.

HZF transcript profiling in wild-type and mutant plants

The *HZF* gene expression levels were determined in 16-day-old wild-type and *hzf* seedlings, subjected to heat stress (38°C for 60 min) conditions. All the seedlings were used for RNA extraction and the transcription profiling was evaluated by RT-PCR, before and during heat stress imposition. RT-PCR analysis showed that the transcripts of *HZF* were strongly induced in the wild-type seedlings, 15 min after the onset of heat stress (Figure 5.12A). The mRNA levels decreased afterwards with the continuation of the heat stress treatment and similar transcript levels to untreated wild-type seedlings

were obtained 60 min after heat stress imposition. When comparing to microarray analysis, the highest levels of *HZF* expression occurred earlier in the heat treatment, since a water bath was used instead of an incubator. These results corroborate the idea that HZF might be involved in the initiation of heat stress response.

The RT-PCR analysis demonstrated that *hzf* mutant was unable to accumulate *HZF* transcripts before and during heat stress treatment (Figure 5.12B), confirming that *hzf* was a null mutant of *HZF*.

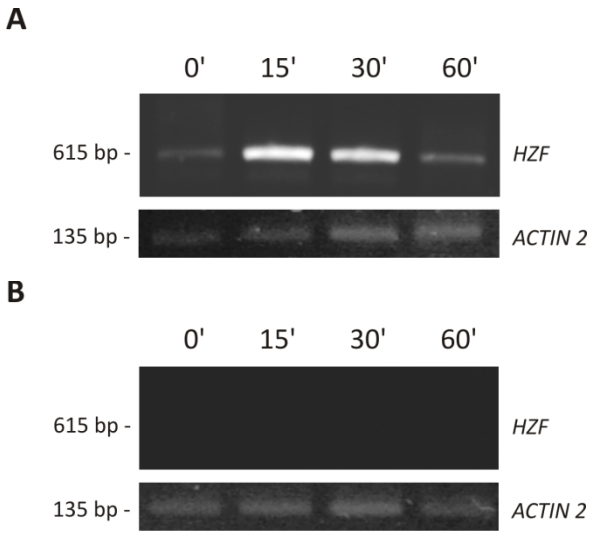


Figure 5.12. RT-PCR analysis of *HZF* transcripts in *Arabidopsis* wild-type Ws (A) and mutant *hzf* (B) seedlings. RNA samples were extracted from 16-day-old seedlings grown in MS agar plates before (0 min) and during heat stress treatment at 38°C for 60 min (at 15, 30 and 60 min). The transcript levels of *ACTIN 2* are displayed as positive controls. Primers sequences are presented in Annex II. The PCR cycles for *HZF* and *ACTIN 2* amplification were respectively 37 and 31 cycles.

Expression vectors construction based on Gateway® technology

A comprehensive analysis of gene function often involves the production of gene fusions aimed for several studies. The production of *HZF* expression vectors for promoter analysis, constitutive expression and subcellular localization studies was performed based on Gateway® cloning technology (Invitrogen). The Gateway® technology is a widely used system for fast and reliable DNA cloning that makes use of the site-specific recombination properties of bacteriophage lambda to promote DNA sequences transfer into multiple vector systems. The fragments of interest are flanked by adapter *att*B sites that promote the recombination with *att*P sites from the donor vector (pDONR™201). Based on a similar recombination strategy, the cloned sequence can then be exported to any of the destination vectors created for functional gene analysis *in planta* (Curtis and Grossniklaus 2003). The main steps of the cloning strategy used in this work are presented in Figure 5.13.

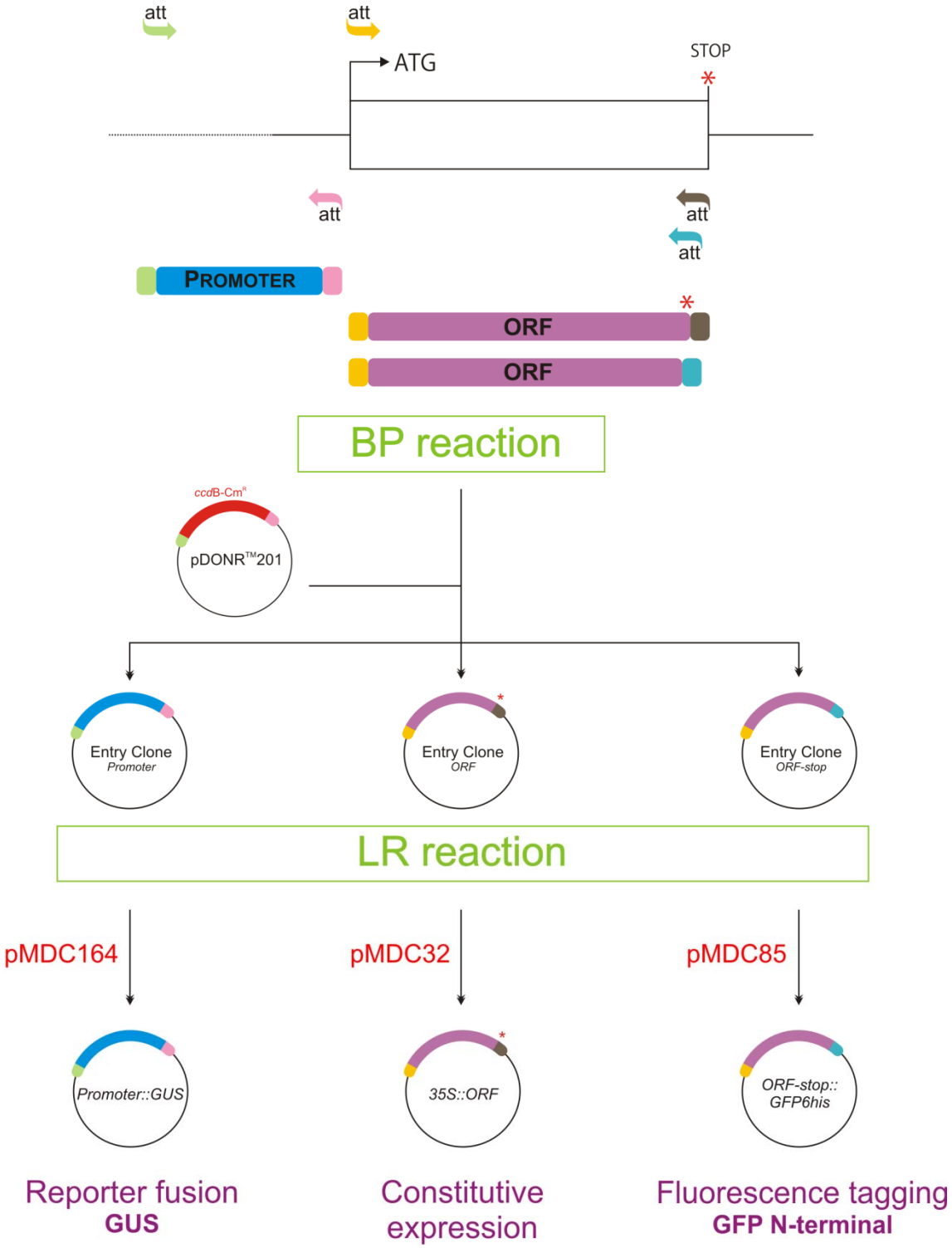


Figure 5.13. Overview of the cloning strategy based on Gateway® technology used for the production of *HZF* constructs. The fragments of interest were amplified by two-rounds of PCR amplification, in which adapter *att*B sites were created on both ends. The fragment of interest was inserted into the donor vector (pDONR™201) by the recombination BP reaction. A subsequent LR recombination reaction promoted the insertion of the DNA sequence into the appropriate destination vector (pMDC vectors). The generated expression clones could then be used in the appropriate expression system.

HZF promoter region (PRM), coding sequence with- (ORF) and without the stop codon (ORF-stop) were amplified and flanked by *att*B regions by two-rounds of PCR amplifications using wild-type (Ws) genomic DNA. Following each PCR amplification, an electrophoretic analysis was performed to confirm the amplification of predicted fragments (Annex I; Figure 5.14A).

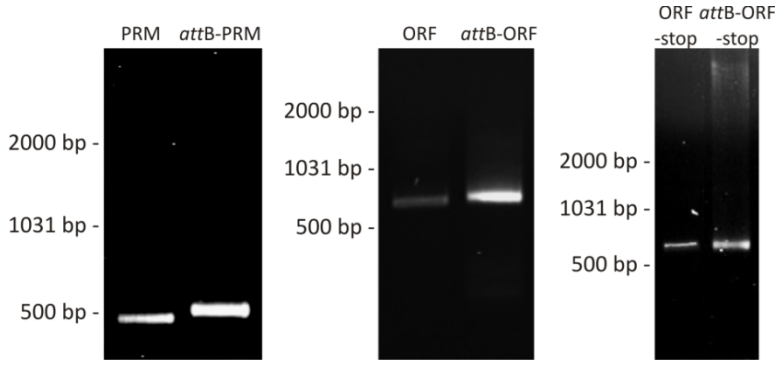
The *att*B-PCR products were introduced into the vector pDONR^{TM}201 (Invitrogen) through the BP recombination reaction. Following transformation of *E. coli* cells with these constructs, transformation efficiency was verified for each insertion by colony PCR (Figure 5.14B). Positive clones were selected according to the expected fragments size (Table 5.3). One entry clone for each cloned fragment (PRM, ORF and ORF-stop) was selected and the inserted sequences were confirmed by sequencing.

Through the LR reaction, the cloned sequences were then transferred to the proper destination vector according to the purpose of the study. Three different constructs were generated: a) for promoter-reporter analysis with GUS, *HZF* promoter sequence was exported to the vector pMDC164; b) for constitutive expression, *HZF* ORF was introduced into the vector pMDC32; and c) for subcellular localization with GFP, an N-terminal fusion was constructed by transferring *HZF* ORF-stop to pMDC85 vector (vector maps on Annex IV). As performed after BP reaction, *E. coli* cells were transformed with the generated vectors and positive clones were identified by colony PCR (Figure 5.14C). The insertion of the sequence of interest was confirmed by sequencing one clone per each construct. For colony PCR and sequencing, specific primers were designed for each destination vector (Table 5.3).

With the construction of the referred transgenes using Gateway® technology, a complete set of genetic tools was created for *HZF* functional characterization. The generated expression vectors were subsequently used to transform *A. tumefaciens* cells aimed for *A. thaliana* transformation. Five bacterial transformants were then selected and verified by colony PCR (Figure 5.14D) using the same specific primers mentioned in Table 5.3.

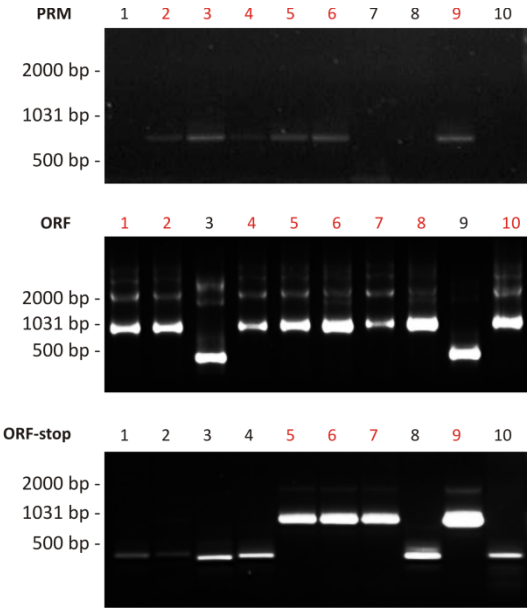
A. tumefaciens containing the *HZF*-based constructs were used to transform wild-type *A. thaliana* Ws plants. The heterozygous T1 plants carrying the desired inserts were selected in hygromycin MS plates. For selection of homozygous plants, T2

A



Expected size (bp)	482	516	623	657	620	654

B



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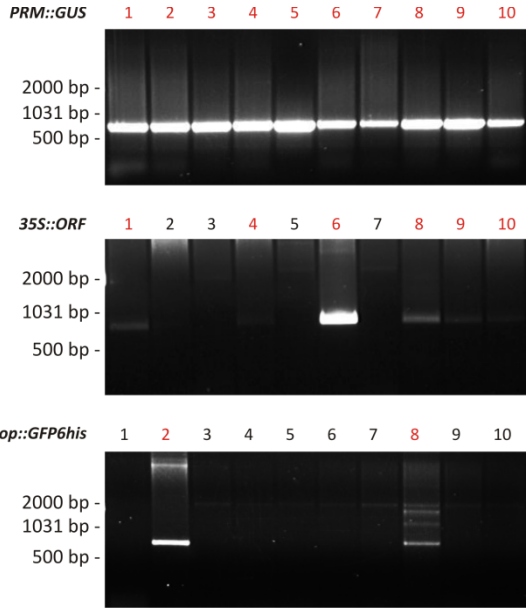

D

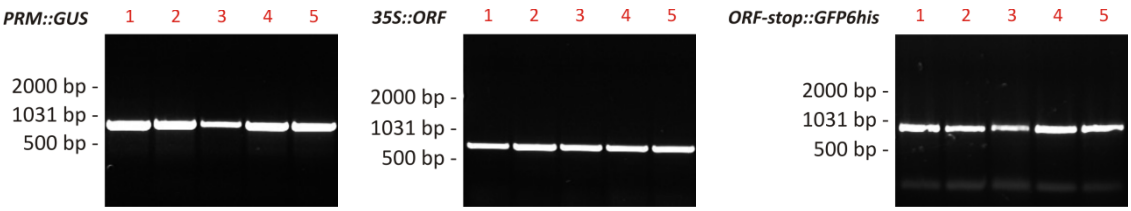

Figure 5.14. Construction of *HZF* expression vectors by Gateway® cloning technology and transformation of *A. tumefaciens* with the generated constructs. Electrophoretic analysis of PCR products for: (A) amplification of the sequences of interest and creation of flanking *att*B sites on its ends; (B) selection of positive *E. coli* transformants carrying the appropriate entry clone after BP reaction; (C) selection of positive *E. coli* transformants carrying the appropriate expression clone after LR reaction and (D) selection of positive *A. tumefaciens* transformants carrying the appropriate expression clone. Primers sequences are presented in Annex II. Primers used and predicted PCR fragments size for B, C and D are presented in Table 5.3. Red numbers indicate the selected clones. PRM: promoter, ORF: open reading frame and ORF-stop: ORF without the stop codon.

Table 5.3 Predicted PCR fragments size after recombination reactions for the construction of *HZF* expression vectors by Gateway® cloning technology.

	After BP reaction			**After LR reaction**		
Insert	*Donor vector*	*Primers*	*Expected size (bp)*	*Destination vector*	*Primers*	*Expected size (bp)*
PRM	*pDONR™201*	*pDONR201 Fo* *pDONR201 Rv*	726	*pMDC164*	*pMDC gus left* *pMDC164 R1 flank*	744
ORF	*pDONR™201*	*pDONR201 Fo* *pDONR201 Rv*	867	*pMDC32*	*pMDC32 R2 flank* *pMDC32 R1 flank*	738
ORF-stop	*pDONR™201*	*pDONR201 Fo* *pDONR201 Rv*	864	*pMDC85*	*pMDC gfp left* *pMDC 35S*	861

plants were selected again in hygromycin MS plates and their seeds will be soon used for selecting T3 plants for further functional studies. The plants containing the *HZF* promoter::GUS constructs will be used to determine tissue-specific expression patterns and to further evaluate the spatial expression pattern of the gene during normal development and after stress conditions. Overexpression lines inducing the constitutive expression of *HZF* (*35S::HZF ORF* constructs) will be used to evaluate the effects caused by enhanced HZF production. *Arabidopsis* wild-type plants transformed with *HZF ORF-stop::GFP6his* fusions will be used to determine the intracellular targeting of HZF protein.

CONCLUSIONS AND FUTURE PERSPECTIVES

Structural analysis performed by *in silico* analysis determined that HZF is a zinc finger family protein containing a conserved C3H2C3-type RING domain. The alignments of gene and protein sequence with other *Arabidopsis* sequences indicated a high degree of similarity with other putative RING finger proteins, which is mainly due to the presence of the conserved C3H2C3-type RING finger domain region. Among the characterized *Arabidopsis* proteins displaying significant similarity, major relevance was given to the ubiquitin ligase CIP8. According to the analysis of *Arabidopsis* RING domains performed by Kosarev *et al.* (2002), the high structural similarity of HZF to CIP8 ubiquitin ligase suggests that HZF is also an E3 ubiquitin ligase. Since a large number of *Arabidopsis* RING domain-containing proteins were found to be able to mediate protein ubiquitination (Stone *et al.* 2005), we hypothesize that HZF may in fact display E3 ubiquitin ligase activity. *In vitro* ubiquitination assays will provide biological evidence of HZF E3 ligase activity.

A linkage between targeted degradation of proteins and temperature stress tolerance was only established for the U-box containing E3 ubiquitin ligase AtCHIP (Yan *et al.* 2003). AtCHIP was found to play an important role in cellular metabolism under both low and high temperature conditions. We are not aware of any RING-type E3 ubiquitin ligase playing a relevant function in the control of heat stress response. In contrast, some examples can be found in *Arabidopsis* concerning other abiotic stresses, as cold, drought and saline stresses (Dong *et al.* 2006; Ko *et al.* 2006; Zhang *et al.* 2007).

Phenotypic analysis showed that *hzf* mutant seeds exhibited delayed germination ability when subjected to heat stress compared to wild-type seeds. These results suggested a putative role of HZF in the switching from dormant to germinating state upon heat stress, but further experimental evidence is still needed. Taking into consideration the putative E3 ubiquitin ligase activity of HZF, we suggested the role of HZF in regulating the levels of a regulator protein implicated in promoting seed dormancy or repressing germination. However, HZF function appears to be only essential upon heat stress, since no significant differences were observed between *hzf* and wild-type germination rates under standard conditions.

According to the *in silico* expression analysis during germination, *HZF* expression is still increased during the first hour of imbibition, reducing greatly afterwards. This pattern suggests that an increase in seed HZF protein content is necessary to promote germination, which is in accordance with the suggested role of HZF in controlling the levels of a dormancy or germination regulator. However, according to the germination rates achieved under normal conditions, HZF function appeared to be unnecessary for inducing germination. These results suggest that a similar protein performing a redundant function may be responsible for promoting germination in *hzf* mutant seeds under standard conditions. The large structural redundancy revealed by the RING domain proteins encoded by the *Arabidopsis* genome, may in fact occult partially or completely the gene function (Kosarev *et al.* 2002). Indeed, only a few mutants of the whole set of *Arabidopsis* RING-finger domains displayed directly informative phenotypes (*e.g.*, COP1, SDIR1 and PRT1).

It is important to notice the considerable high similarity of HZF to one of the uncharacterized RING-containing protein (L-HZF), indicated by gene and protein alignments. This protein had also been included in the same cluster of HZF and CIP8 in the study performed by Kosarev *et al.* (2002), and probably has a similar function to HZF (*i.e.*, E3 ubiquitin ligase). The high degree of *HZF* co-expression with *L-HZF* also has suggested that these proteins may possibly have redundant function. When comparing the gene expression analysis of *L-HZF* to *HZF*, parallel results were obtained (Annex V and Figure 5.6). The gene *L-HZF* was found to be highly expressed during the same development stages of wild-type *Arabidopsis* as *HZF*, although strongest expression levels were predicted. This gene is also strongly expressed during cotyledon greening and maturation of flowers and pollen. In seeds, similarly to *HZF*, the expression levels of *L-HZF* are increased up to 1 h of seed imbibition followed by an evident decrease. Also during imbibition, higher expression levels were predicted for *L-HZF* when compared to *HZF*, suggesting that L-HZF may be mostly involved in plant development. Microarray expression data of *L-HZF* under heat stress (38°C for 3 h) was also compared to *HZF* expression using the *heat stress time course* experiment, included in the *AtGenExpress abiotic stress series* (Annex VI) (Kilian *et al.* 2007). A similar response profile was observed for *L-HZF*, reaching highest expression levels one hour after heat stress imposition, followed by a gradual decrease. However, *HZF* expression was

induced at higher levels (mainly in shoots and cell suspensions), which suggests the main implication of HZF in heat responses. It is then possible that L-HZF acts redundantly with HZF, which explains why similar germination rates were achieved for mutant and wild-type seeds under normal conditions. Having a similar function, L-HZF might be mainly involved in normal plant development, while HZF is more important when heat stress conditions are present. Under normal conditions, L-HZF may be the major responsible for promoting the germination process, although HZF may perform a similar function. However, HZF is essential for assuring the induction of normal germination upon heat stress conditions, being the key regulator of the germination process.

The regulation of different biological processes (drought response and gametogenesis) has recently been demonstrated to be performed by redundant pairs of RING-type E3 ubiquitin ligases (Liu *et al.* 2008; Qin *et al.* 2008). In these processes, an E3 ligases pair seems to be responsible for turning off the key regulator of the process by ubiquitin mediated-proteolysis. The redundant control of proteolysis was considered to be advantageous, since the phenotypic consequences were only observed when both proteins fail to degrade the regulatory proteins. In agreement with this work, it is reasonable to hypothesize that the levels of a key protein regulator of germination/dormancy processes can be controlled by the redundant pair of E3 ubiquitin ligases (HZF/L-HZF).

The ABA levels have major effect on seed dormancy and therefore the process of germination is partially controlled by ABA signaling (Bentsink and Koornneef 2008). ABA levels have been shown to decrease at the end of seed maturation and during imbibition due to the activity of ABA catabolism enzymes. Increased ABA content allows to maintain seed dormancy. Higher levels of ABA are achieved through an active process involving *de novo* ABA synthesis that remains active until favourable conditions are available for germination. Recently, RING E3 ubiquitin ligases have been implicated in targeting specific components of ABA signaling pathway to degradation, thereby altering seed dormancy and germination (reviewed by Bu *et al.* 2009). Considering the phenotypic delay in germination observed in *hzf* seeds under heat stress, HZF probably also acts as a regulator of ABA signaling. It would be interesting to further investigate this putative involvement of *HZF* in the regulation of ABA signaling.

The results hereby presented and the discussed considerations led to the proposition of a model of HZF action under standard and heat stress-imposed conditions (Figure 5.15). In standard conditions, the transcripts of *HZF* and mainly *L-HZF* are present in high levels in dry wild-type seeds, increasing their levels upon seed imbibition. Both proteins act redundantly in degrading a regulator protein controlling seed dormancy/germination (putatively also involved in ABA signaling). As a result of ubiquitination and degradation of this key protein, ABA signaling is potentially deactivated and seeds are able to germinate. Although *HZF* transcripts fail to accumulate in the mutant, *hzf* seeds germinate in standard conditions as well as the wild-type seeds, since L-HZF is present at high levels. Under heat stress treatment, both genes are induced in wild-type seeds (although *HZF* expression is predicted to be stronger than *L-HZF*) and germination can proceed whenever temperatures allow it. In mutant seeds, only *L-HZF* expression is induced after heat treatment, but this induction would be not sufficient to promote germination as rapid as in wild-type seeds. Nonetheless, L-HZF can mediate targeted proteolysis, but their low levels result in delayed germination.

Although this model could relate the gene redundancy with delayed germination of mutant seeds upon heat stress, it should be noted that gene expression of both genes (*HZF*/*L-HZF*) is greatly reduced a few hours after seed imbibition. Therefore, when we have applied the heat stress on seeds (72 h after seed imbibition) the gene expression of both genes was almost certainly extremely reduced and low levels of transcripts would be expected. Therefore, the determination of HZF and L-HZF protein levels during seed imbibition and germination is mandatory to confirm the proposed model. The effect of heat on germination of wild-type and mutant seeds could be also related with the thermostability of both proteins.

Microarray data presented in Chapter 3 indicated that *HZF* expression was induced to high levels 1 h after heat stress treatment (Figure 3.12A), suggesting the involvement of HZF in the initial phase of heat stress response. Also, RT-PCR analysis in wild-type seedlings showed the strongest accumulation of *HZF* transcripts during the

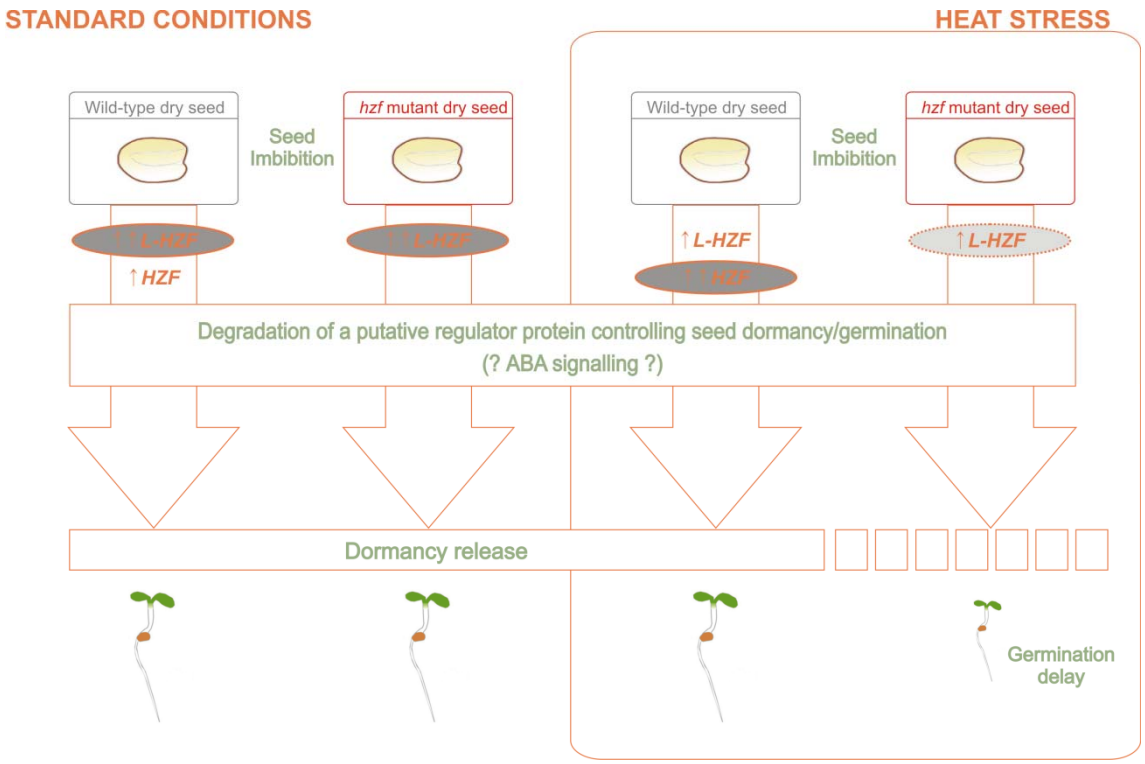

Figure 5.15. Model representation for explaining the germination delay in *hzf* mutant seeds under heat stress conditions. The redundant pair HZF/L-HZF is suggested to be involved in controlling the levels of a regulator protein essential for maintenance of seed dormancy or inhibition of germination under standard and heat stress-imposed conditions (see text for details).

first minutes (15 min) after heat stress imposition (38°C). The network of co-expressed genes corroborates this hypothesis by the substantial number of heat shock transcription factors identified. As already mentioned, the initiation of heat stress response depends greatly on the action of HSFs, which mediate HSPs expression and activate other signaling cascades (*e.g.*, production of antioxidants). In addition to dormancy/germination regulation, we cannot exclude the possibility that HZF may be involved in the control of HSF signaling pathway, due to its putative ubiquitin ligase activity. A dual function of HZF as a regulator of dormancy/germination processes and HSF signaling should be considered.

Functional characterization of *HZF* was just barely initiated. The present work provided evidences for the involvement of HZF in regulating the germination process under heat stress imposition. Further biochemical analysis is necessary to ascertain HZF role as a putative E3 ubiquitin ligase involved in the heat stress response. Additional studies must be performed in order to provide answers to the several remaining open questions, particularly regarding the existence of gene redundancy

and the potential role in repressing dormancy or promoting germination. Results from the ongoing Southern blot analysis, as well as complementation and gain-of-function studies will also provide evidences that the mutant phenotype observed at the seed level was due only to the disruption of *HZF*. *In situ* expression analysis, which will be performed as soon as T3 transgenic plants are available, will provide helpful insights into further characterization of *HZF*.

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ANNEX I

Standard protocols

Isolation of genomic DNA from *Arabidopsis*

Single leaves were placed in microcentrifuge tubes and ground to a powder with liquid nitrogen. To each tube, 700 µl of CTAB extraction buffer [2% (w/v) hexadecyltrimethylammonium bromide; 0.1 M Tris-HCl, pH 8.0; 1.4 M NaCl; 0.02 M EDTA; 0.1% (v/v) 2-mercaptoethanol] were added. Following an incubation of 65°C for 25 min, an equal volume of chloroform:isoamilic acid [24:1 (v/v)] was added. The tube was gently inverted and centrifuged in a microcentrifuge for 5 min. Genomic DNA was precipitated by addition of 1 volume of cold isopropanol and centrifugation for 20 min. Precipitated DNA was washed by addition of 300 µl of ethanol and recovered by centrifugation. Finally, the DNA was resuspended in 50 µL of TE buffer [5 mM Tris-HCl, pH 8.0; 1 mM EDTA] containing 100 $\mu g.ml^{-1}$ of RNase and stored at 4°C.

PCR amplification

Amplification of DNA fragments of interest was performed by PCR as described by Mullis and Faloona (1987). PCR reactions were prepared as presented in Table A.

Table A Components used for the preparation of PCR reactions in standard/colony PCR and for cloning strategies.

	Standard PCR/Colony PCR	PCR for cloning
DNA template	1 µg/Colony*	600 ng
Taq DNA polymerase (1 U)	0.25 µl	25 µl ACCUZYME Mix**
5x Buffer	10 µl	
2 mM dNTPmix	5 µl	
25 mM $MgCl_2$	4 µl	
Primers [stock] = 10 pmol/µl	2 µl (each)	2 µl (each)
Millipore H_2O	up to 50 µl	up to 50 µl

* *E. coli*: a single colony was picked with an autoclaved pipette tip and swirled into the PCR mixture
A. tumefaciens: a bacterial colony was previously incubated in 20 mM NaOH at 37°C for 10 min and 3 µl were used for PCR
** ACCUZYME™ Mix (Bioline): 120 mM Tris-HCl, pH 8.3; 12 mM $(NH_4)_2SO_4$; 20 mM KCl; 4 mM $MgSO_4$; 2 mM dNTPs; stabiliser; ACCUZYME™ DNA polymerase

Separation of fragments on agarose gel electrophoresis

DNA fragments were separated in agarose gel [0.8-1.5% (w/v), depending on fragments size] prepared with TAE buffer [40 mM Tris; 20 mM acetic acid; 1 mM EDTA, pH 8.0] and 0.1 $\mu g.ml^{-1}$ ethidium bromide. DNA samples were premixed with 0.2 volumes of 6x MassRuler™ DNA Loading Dye [10 mM Tris-HCl, pH 7.6; 0.03% (w/v) bromophenol blue; 60% (v/v) glycerol; 60 mM EDTA], supplied with the molecular weight marker used for sizing the DNA fragments (Fermentas). The ready-to-use MassRuler™ DNA Ladder Mix (Fermentas) was directly loaded onto the agarose gel. Electrophoresis was performed

at 50-100 V using TAE buffer in a horizontal electrophoresis system and the fragments were visualized on a transilluminator under UV light (254 nm).

For RNA electrophoresis, samples were denatured at 65°C for 5 min, prior to being loaded on the gel and separation occurred under the same conditions.

Isolation of total RNA from *Arabidopsis* by the TRIzol method

TRIzol® reagent [380 $ml.l^{-1}$ phenol; 0.8 M guanidine thiocyanate; 0.4 M ammonium thiocyanate; 0.1 M NaOAc, pH 5; 50 $ml.l^{-1}$ glycerol] (Invitrogen) was added, in the proportion of 1 ml to 50-100 mg of frozen plant tissue ground in liquid nitrogen. After homogenization, samples were incubated at room temperature for 5-10 min and 0.2 volumes of chloroform were subsequently added. The mixture was then vigorously mixed for 15 sec. The separation of phases was achieved after incubation at room temperature for 2-3 min and centrifugation at 12000 *g*, for 15 min, at 4°C. Isopropanol (0.5 volumes) was added to the aqueous phase and the mixture was incubated at room temperature for 10 min. RNA precipitation occurred by centrifugation at 12000 *g*, for 10 min, at 4°C, and the pellet was rinsed with 75% (v/v) ethanol. An additional centrifugation step was performed (7500 *g*, 5 min, at 4°C) and the RNA was then air dried for 5-10 min. The resultant pellet was resuspended in 30 µl RNase-free water and completely dissolved by incubation at 55-60°C for 10 min. RNA was immediately used or stored at -80°C.

DNA and RNA quantification and purity

Nucleic acids were evaluated in a spectrophotometer (Nanodrop), measuring absorbance (A) values at 260, 280 and 230 nm. DNA or RNA concentration was calculated considering that an $A_{260\,nm}$ reading of 1 is equivalent to about 50 µg DNA/ml or 40 µg RNA/ml. Nucleic acids purity was determined by $A_{260\,nm}/A_{280\,nm}$ and $A_{260\,nm}/A_{230\,nm}$ ratios, which should be very close to 2.0, and by fractioning DNA/RNA samples in an agarose gel.

Cloning associated protocols - Gateway® technology

Purification of DNA by polyethylene glycol precipitation

The *att*B-PCR fragments were purified by size-selective precipitation with PEG (Lis 1980; Paithankar and Prasad 1991). Three volumes of TE buffer [5 mM Tris-HCl, pH 8.0; 1 mM EDTA] and 2 volumes of PEG/$MgCl_2$ solution [30% (w/v) PEG 8000; 30 mM $MgCl_2$] were added to the PCR sample. Immediately after mixing, DNA was precipitated in a microcentrifuge for 20 min, at room temperature. After complete removal of supernatant, DNA was resuspended in 30 µl of TE buffer.

Gateway® recombination reactions

Gateway® BP and LR recombination reactions were prepared as presented in Table B, according to Invitrogen instructions for a final volume of 10 µl. Both reactions were incubated overnight at 25°C.

Table B Components used for the preparation of BP and LR recombination reactions (Invitrogen).

	***HZF* promoter**	***HZF* ORF**	***HZF* ORF-stop**
BP reaction			
*att*B-PCR product	7.3 µl	7.3 µl	7.3 µl
pDONR^{TM}201	0.7 µl	0.7 µl	0.7 µl
TE buffer	-	-	-
BP Clonase^{TM} II Enzyme Mix	2 µl	2 µl	2 µl
LR reaction			
Entry vector (150 ng)	1.5 µl	1.5 µl	1.5 µl
Destination vector (150 ng.µl^{-1})	1 µl	1 µl	1 µl
TE buffer	7.5 µl	7.5 µl	7.5 µl
LR Clonase^{TM} II Enzyme Mix	2 µl	2 µl	2 µl

TE buffer: 5 mM Tris-HCl, pH 8.0; 1 mM EDTA

Preparation and transformation of *E. coli* cells by *SEM*

Preparation and transformation of chemically competent *E. coli* cells was performed as described by Inoue *et al.* (1990). A single colony of *E. coli* strain XL1-Blue (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (*Tet*^{r})]) (Bullock *et al.* 1987) was inoculated into 250 ml of SOB medium [0.5% (w/v) yeast extract; 2% (w/v) tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; pH 7.5] and incubated overnight at 18°C with vigorous shaking (200-250 rpm). When $A_{600\,nm}$ reached 0.6, the *E. coli* culture was placed on ice for 10 min and the cells were harvested by centrifugation at 2500 *g*, for 10 min, at 4°C. The pellet was resuspended in 80 ml of ice-cold previously filtered TB [10 mM Hepes; 15 mM $CaCl_2$; 250 mM KCl; pH 6.7 using KOH; 55 mM $MnCl_2$ added after pH adjustment] and incubated on ice for 10 min. A centrifugation in identical conditions (2500 *g*, for 10 min, at 4°C) was performed and cells were gently resuspended in 20 ml TB containing 7% (v/v) DMSO. After 10 min of ice incubation, the cell suspension was divided into 100 µl aliquots, which were immediately frozen in liquid nitrogen and stored at -80°C.

DNA was gently mixed with an aliquot of ice-thawed competent cells and incubated for 30 min on ice. Cells were heat shocked at 42°C for 90 sec, with gentle agitation, and instantly incubated on ice for 2 min. One milliliter of SOC medium [SOB medium, containing 20 mM glucose] was added to the mixture. After incubation at 37°C for 1 h, with vigorous shaking, cells were harvested by centrifugation at 12000 *g* for 2 min and the pellet was resuspended in 100 µl of supernatant. Cells were then plated on the appropriate selective medium and incubated overnight at 37°C.

Isolation of plasmidic DNA from *E. coli*

Plasmidic DNA from *E. coli* was isolated at small-scale using GenElute^{TM} HP Plasmid Miniprep Kit (Sigma), according to supplier instructions. *E. coli* cells were previously cultured overnight at 37°C in LB medium supplemented with 50 µg.ml^{-1} kanamycin. Small-scale purifications were prepared for sequencing and cloning reactions.

Preparation and transformation of *A. tumefaciens* cells

A single colony of *A. tumefaciens* strain EHA105 (Hood *et al.* 1993) was inoculated into 10 ml LB medium [1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract; pH 7.0], supplemented with 50 $\mu g.ml^{-1}$ rifampycin. Following overnight incubation at 30°C, with continuous shaking, the culture was diluted (50 µl of the starter culture in 50 ml of fresh medium) and growth proceeded under the same conditions until $A_{600\,nm}$=0.5-1.0. After being cooled on ice for 10 min, *A. tumefaciens* cells were harvested by centrifugation at 3000 *g* for 6 min, at 4°C. The pellet was then rinsed with 1 ml of recently sterilized ice-cold 20 mM $CaCl_2$ and cells were centrifuged again in the same conditions (3000 *g* for 6 min, at 4°C). After being resuspended in 1 ml of the same solution, 100 µl of *A. tumefaciens* cells were used for transformation. Following addition of DNA (5 µg) and gentle mixing, the mixture was frozen in liquid nitrogen for 5 min. After thawing for 10 min at room temperature, 1 ml of LB medium was added and cells were incubated at 30°C, for 3 h, with continuous shaking. Selection was performed on LB medium supplemented with 50 $\mu g.ml^{-1}$ rifampycin and the appropriate antibiotic after incubation overnight at 30°C.

Transformation of *Arabidopsis* plants by floral dip method

A. tumefaciens clones carrying the appropriate construct were inoculated into 7 ml of LB medium [1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract; pH 7.0] supplemented with 50 $\mu g.ml^{-1}$ kanamycin and 50 $\mu g.ml^{-1}$ rifampycin. Growth proceeded overnight at 28°C, with continuous shaking. *A. tumefaciens* culture was diluted 100x in 200 ml of LB medium, pH 5.4, supplemented with 19.6 $\mu g.ml^{-1}$ acetosyringone and 50 $\mu g.ml^{-1}$ kanamycin. This culture was further cultivated overnight at similar conditions. *A. tumefaciens* cells were recovered by centrifugation at room temperature (5000 rpm, 12 min) and resupended in 250 ml of 5% (w/v) sucrose, containing 125 µl of 0.05% (v/v) Silwett L-77. This suspension will be used for transforming *Arabidopsis* wild-type plants at a density of five plants per pot. The plants which had been grown until early bolting (10-15 cm) and had any visible siliques removed just before transformation, were dipped into the solution for 20 sec. Transformed plants were placed horizontally in a tray covered with plastic. After one day in the dark, plants were transferred to the growth room, under standard conditions.

ANNEX II

The oligonucleotides (Table C) were synthesized by Metabion or Frilabo services. Stock primers solutions were prepared to a final concentration of 100 µM in Millipore water, according to suppliers instructions. A working solution of 10 µM was used for PCR amplification and sequencing.

Table C Oligonucleotides used in this work for each purpose. The recombinant Gateway sequences are underlined.

	Sequence (5' → 3')
Genotyping for *hzf* mutants (FLAG)	
T-DNA left border 4 (Lb4)	CGTGTGCCAGGTGCCCACGGAATAGT
HZF dpcr LP	TCCACTAGGGAGTGAATCGTG
HZF dpcr RP	GGGTTTGATGAGGATTATGGA
***HZF* promoter amplification**	
HZF GA1	AAAAAGCAGGCTGATAAATAACAATAGGAGCATAT
HZF GB1	AGAAAGCTGGGTGCAAACAAACTATTGTTTTGAAG
***HZF* ORF amplification**	
HZF GC1	AAAAAGCAGGCTTTGCGATGGCGACAGAACAAG
HZF GD1	AGAAAGCTGGGTCCTAATTCTCGGACTCAACACCAT
HZF GE1 (without stop codon)	AGAAAGCTGGGTAATTCTCGGACTCAACACCATCACT
Gateway® BP entry primers (*att*B adapters)	
*att*B1 (Fo)	GGGGACAAGTTTGTACAAAAAAGCAGGCT
*att*B2 (Rv)	GGGGACCACTTTGTACAAGAAAGCTGGGT
Verification of cloning	
pDONR201 Fo	TCGCGTTAACGCTAGCATGGATCTC
pDONR201 Rv	GTAACATCAGAGATTTTGAGACAC
pMDC gus left	CAAGGCGATTAAGTTGGGTAAC
pMDC164 R1 flank	CAGACTGAATGCCCACAGG
pMDC32 R2 flank	CGGCCGCTCTAGAACTAGTTAA
pMDC32 R1 flank	ACTCTAGAGGATCCCCGGGTA
pMDC gfp left	TTGGGACAACTCCAGTGAAAAG
pMDC 35S	TTCATTTCATTTGGAGAGGACC
RT-PCR	
ACT2 RT F	GACCTTGCTGGACGTGACCTTAC
ACT2 RT R	GTAGTCAACAGCAACAAAGGAGAGC
HZF RT F	ATGGCGACAGAACAAGAAGCTG
HZF RT R	CTAATTCTCGGACTCAACACCATC

ANNEX III

Amplifications by PCR were performed using Mastercycler Gradient (Eppendorf) under the conditions described below (Table D).

Table D PCR conditions used in this work for each purpose.

	Temperature (time)	Number of cycles
Genotyping for *hzf* mutants (FLAG)		
Initial denaturation	94°C (5 min)	
Denaturation	94°C (45 sec)	30
Annealing	55°C (45 sec)	
Extension	72°C (1 min)	
Final extension	72°C (10 min)	
Gateway® cloning – first PCR		
Initial denaturation	94°C (5 min)	
Denaturation	94°C (30 sec)	35
Annealing	55°C (30 sec)	
Extension	72°C (1 min)	
Final extension	72°C (3 min)	
Gateway® cloning – second PCR		
Initial denaturation	94°C (5 min)	
Denaturation	94°C (30 sec)	5
Annealing	45°C (30 sec)	
Extension	72°C (1 min)	
Denaturation	94°C (30 sec)	20
Annealing	55°C (30 sec)	
Extension	72°C (1 min)	
Final extension	72°C (3 min)	
Colony PCR for verification of transformants		
Initial denaturation	94°C (10 min)	
Denaturation	94°C (45 sec)	30
Annealing	55°C (45 sec)	
Extension	72°C (45 sec)	
Final extension	72°C (10 min)	
RT-PCR		
Initial denaturation	94°C (5 min)	
Denaturation	94°C (40 sec)	37^{1} or 31^{2}
Annealing	55°C (40 sec)	
Extension	72°C (90^{1} or 30^{2} sec)	
Final extension	72°C (5 min)	

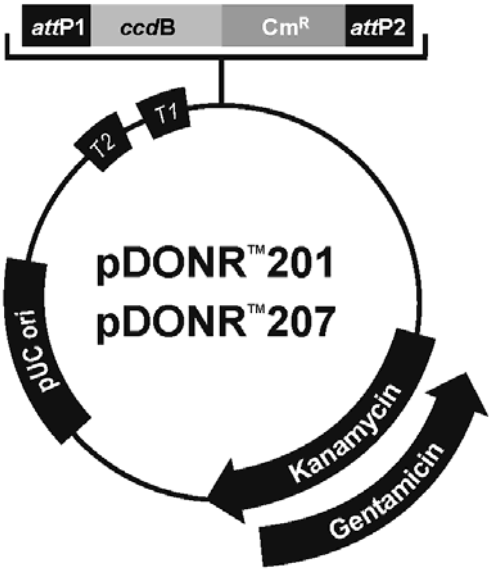
[1] *HZF*

[2] *ACTIN 2*

ANNEX IV

Vector maps

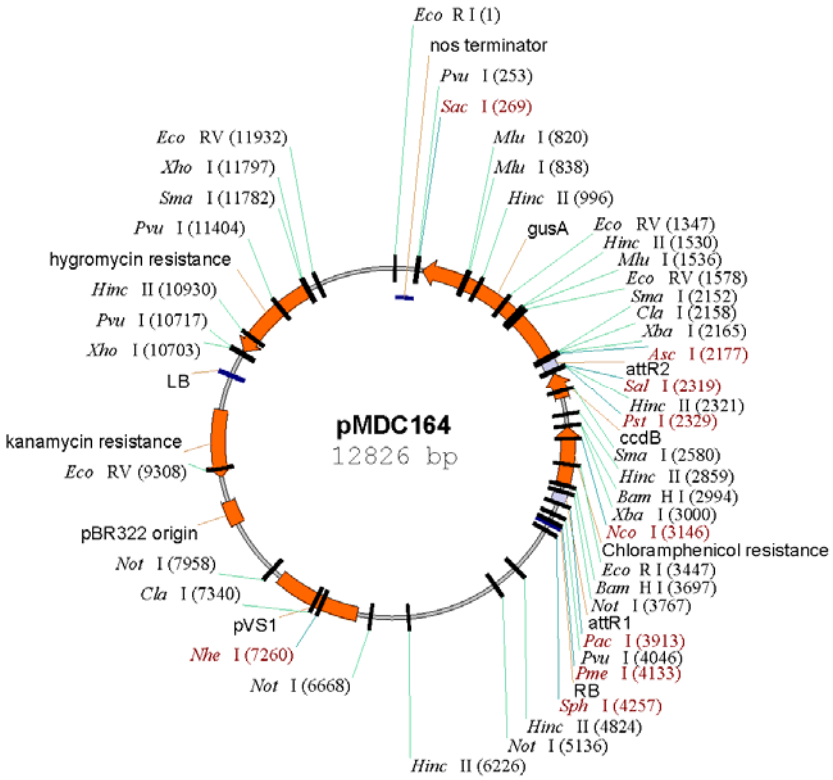
pDONR™201



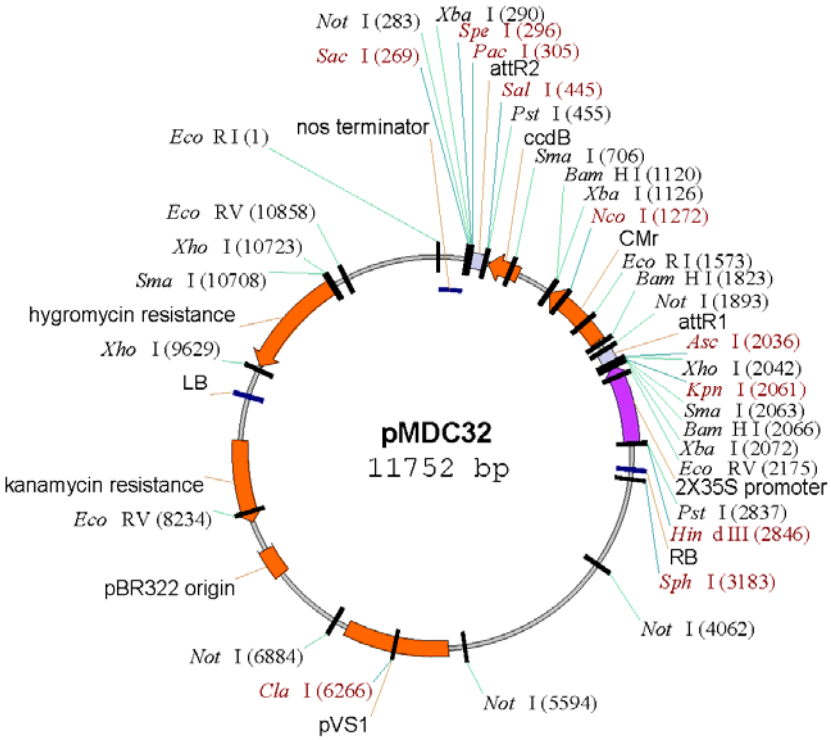
Comments for:	pDONR™201 4470 nucleotides
*rrn*B T2 transcription termination sequence (c):	73-100
rrnB T1 transcription termination sequence (c):	232-275
Recommended forward priming site:	300-324
*att*P1:	332-563
*ccd*B gene (c):	959-1264
Chloramphenicol resistance gene (c):	1606-2265
*att*P2 (c):	2513-2744
Recommended reverse priming site:	2769-2792
Kanamycin resistance gene:	2868-3677
Gentamicin resistance gene (c):	---
pUC origin:	3794-4467

(c) = complementary strand

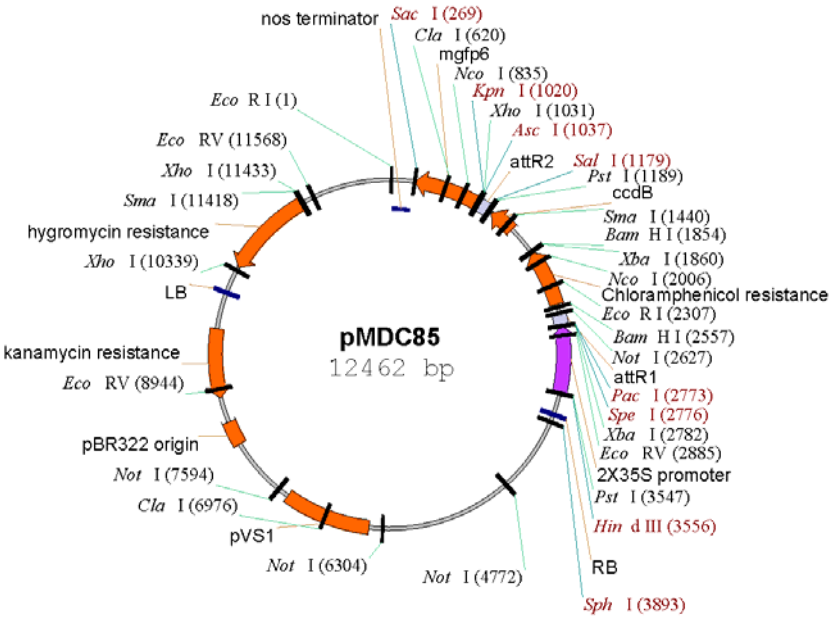
pMDC164



pMDC32



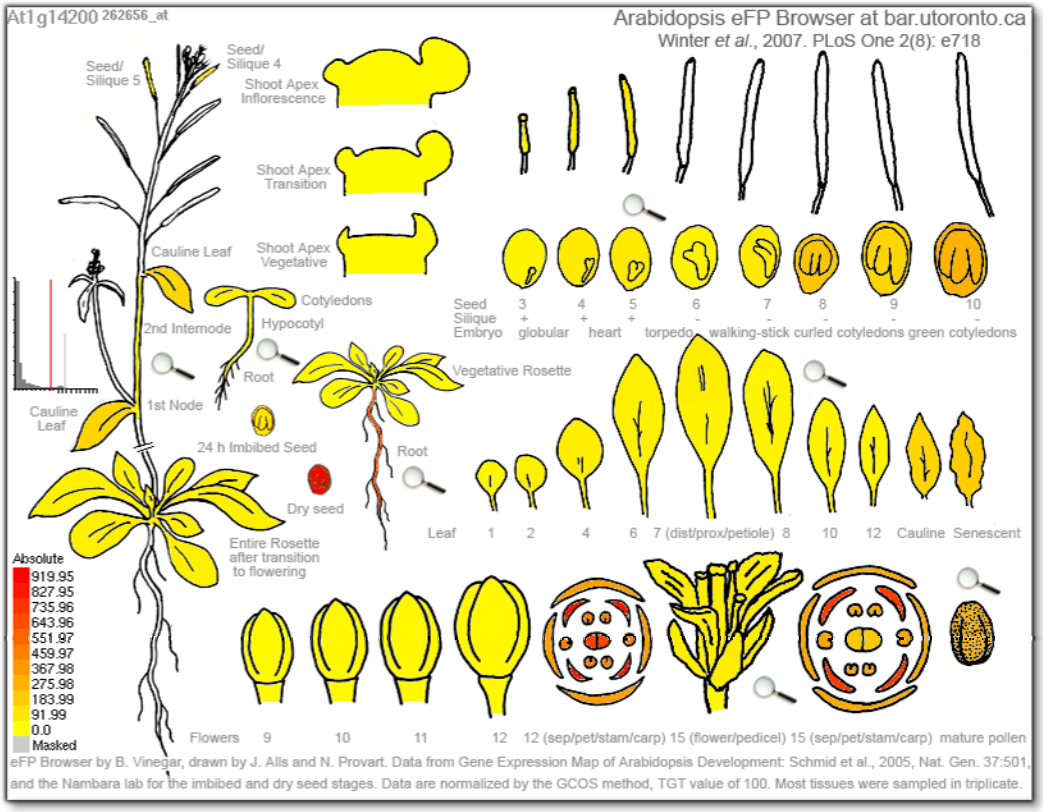
pMDC85



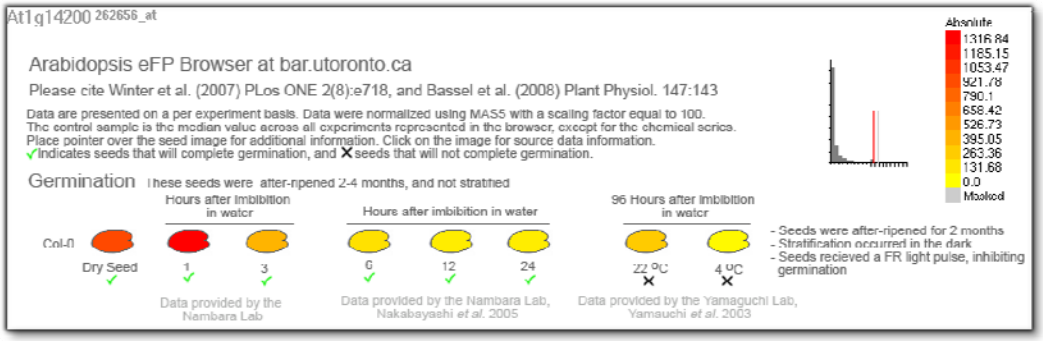
ANNEX V

Gene expression pattern of *L-HZF* gene as an "electronic fluorescent pictographic" representation, during development of *A. thaliana* wild-type tissues (A) and seed germination (B). Expression levels are identified in colors as depicted in the bars. This analysis was performed using Arabidopsis eFP Browser provided by BAR, considering the Development Map (A) and Seed (B) data sources in the absolute mode.

A

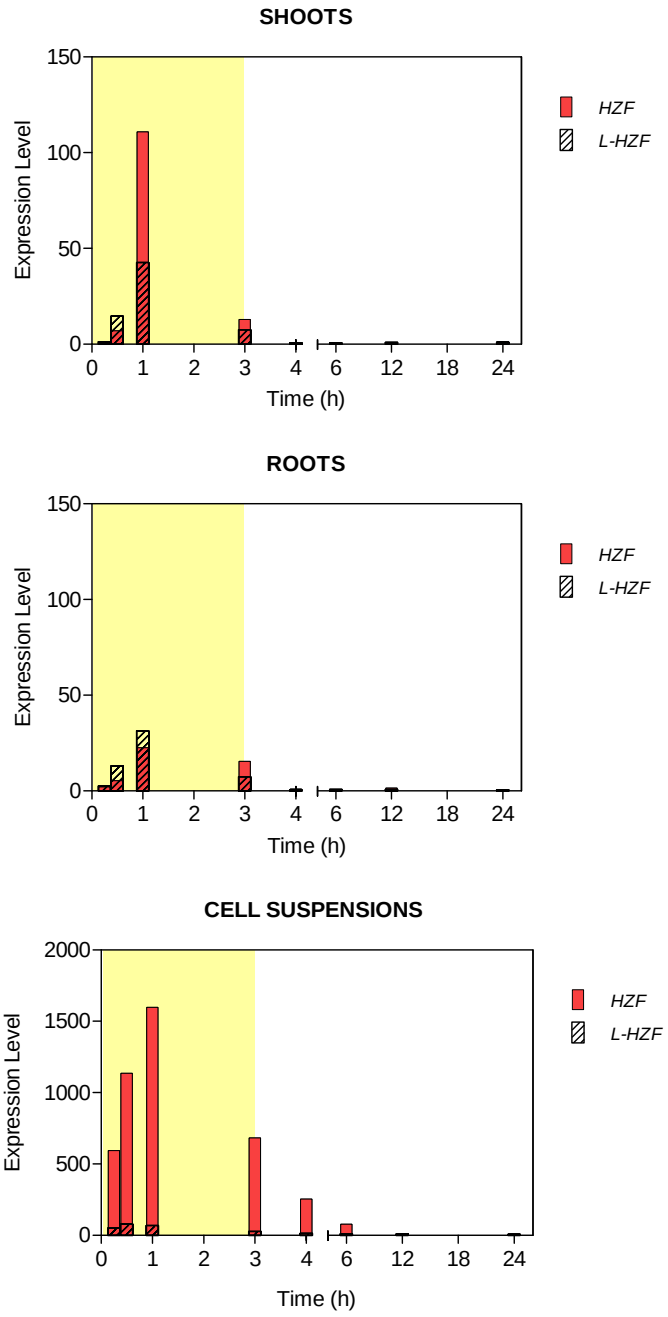


B



ANNEX VI

Expression response profiles of *HZF* and *L-HZF* genes on *A. thaliana* shoots, roots and cell suspensions after a heat stress (38°C) treatment. The data was obtained from the *heat stress time course* experiment, included in the *AtGenExpress abiotic stress series* (Kilian *et al.* 2007). After 3 h of light treatment, 16-day-old plants or 6-day-old cell suspensions were subjected to a heat stress of 38°C for 3 h (highlighted in yellow) and were then allowed to recover at 25°C. Expression levels are displayed as relative values (pixel count in heat stressed cells/pixel count in control cells).



Chapter 6

Final Considerations

CONTENTS

CONCLUDING REMARKS

The main objective of the studies presented in this thesis was to contribute for a better understanding of the physiological and molecular mechanisms underlying plant adaptation to heat stress. For our purpose, the surviving ability of *Populus euphratica* suspended cells was evaluated after different temperature treatments. The high natural tolerance to extreme temperatures of *P. euphratica* (-45°C to +54°C) was not clearly reproduced by the suspended cells. *P. euphratica* cell suspensions were able to tolerate heat treatments up to 45°C for 20 min. The higher sensibility of suspended cells in sensing a temperature increase, when compared to whole plants in their natural environment, probably results in a more intense and unspecific response. Appropriate tissue integration was suggested to be essential for plant cells to achieve the capacity to cope with a heat stress situation. Although further studies are required, the preliminary evaluation of the occurrence of PCD and production of ROS suggested that both processes were not implicated in the induction of the observed cell death.

The use of publicly available expression data allowed the evaluation of the transcriptome of heat-stressed *Arabidopsis thaliana* shoots, roots and cell suspensions. Similarly to *P. euphratica* studies, suspended cells were hypersensitive to heat stress treatment, which have resulted in a massive number of up-regulated genes when compared to shoots and roots. However, these genes may reflect a more general reaction rather than a heat stress-specific response. The work performed in both species allowed to assume that cell suspensions are not the most suitable system to study heat stress responses in plants. Although they could be extremely useful in studies where cell homogeneity and reduced complexity are desired, special attention should be taken when studying heat-associated responses. The thermotolerance mechanisms activated by increased temperatures *in vivo* are probably induced at much lower temperature levels in suspended cells, and thus heat tolerance assays should be performed at lower temperature treatments.

The main purpose of the work performed in *A. thaliana* was the identification and functional characterization of novel determinant genes involved in thermotolerance. Regarding the identification of stress determinants, a

straightforward reverse genetics strategy was proposed based on the tools and resources currently available for *Arabidopsis* functional studies. The identification of potential candidates contributing to thermotolerance was successfully achieved by using the outlined strategy. The two heat-induced identified genes, *HZF* and *HRR*, were preliminarily characterized by collecting relevant information on web-based tools and resources. Both genes seem to be putatively involved in the regulation of the heat stress response at different stages. This hypothesis would only be confirmed by further functional characterization. The functional characterization of *HZF* was initiated in this work, while the functional studies on the other selected gene (*HRR*) is currently being performed in our laboratory. HZF was found to be a zinc finger family protein with a conserved C3H2C3-type RING domain putatively involved in protein ubiquitination. The most similar protein to HZF, encoded by *L-HZF* gene, was suggested to have a redundant function. Besides presenting high nucleotidic and amino acidic sequence similarity, this gene had been previously indicated as a potential redundant gene of *HZF* in clustering analysis of *Arabidopsis* RING-finger domains. Also, *HZF* and *L-HZF* were predicted to be highly co-expressed and to have a similar expression pattern during development of *Arabidopsis* wild-type tissues and seed germination. This *in silico* analysis suggested that an increase in HZF and L-HZF protein content during seed imbibition is essential to promote germination. However, higher expression levels were predicted for *L-HZF* under standard conditions when compared to *HZF*. During heat stress imposition, both genes depict a similar response profile, but *HZF* is induced at higher levels as observed by analysis of microarray data. These results suggested that HZF appears to be mainly implicated in heat responses, whereas L-HZF might be essentially involved in plant development under standard conditions.

The establishment of gene function by mutant phenotype analysis greatly depends on the assay conditions. Aiming for the setting up of a standard approach for the detection of heat stress-associated phenotypes in *Arabidopsis* knockout mutants, several protocols relying on the evaluation of germination rate and seedling survival were established. The proposed protocols, comprising variable temperatures and periods of heat treatment, were initially used to characterize basal and acquired thermotolerance of *Arabidopsis* wild-type plants. The established assay conditions allowed the successful detection of heat-associated phenotypes in two mutants

(*hot1-3* and *atrbohD*), previously identified as thermotolerance defective. The moment of germination assessment was found crucial for detecting phenotypic alterations in germination assays. Therefore, for the detection of germination-associated phenotypes, a time-course evaluation of the number of germinated seeds should be performed.

Phenotypic analysis of *hzf* knockout mutants helped to reveal HZF involvement in the regulation of seed dormancy/germination under heat stress conditions. The delayed germination observed in *hzf* mutant seeds subjected to heat stress, when compared to wild-type, was suggested to result from impaired ubiquitination of a regulator protein implicated in maintaining seed dormancy or inhibiting germination. However, under standard conditions HZF did not seem to be essential, which could be due probably to the existence of gene redundancy with *L-HZF*. A model of HZF function under standard and heat-stressing conditions was proposed. In standard conditions, the high transcripts levels of *HZF* and mainly *L-HZF* present in imbibed seeds are responsible for redundant ubiquitination of a regulator protein controlling seed dormancy/germination. The degradation of this key regulator, which could also be involved in ABA signaling, results in dormancy release and seed germination. The *hzf* mutant seeds also germinate in standard conditions due to the action of high levels of L-HZF. Upon heat stress, wild-type seeds accumulate both transcripts (but strongly *HZF*) which lead to germination whenever temperatures allow it. In *hzf* mutant seeds, only *L-HZF* expression is induced after heat treatment, but the low levels of L-HZF are not sufficient to ensure that germination proceeds as rapid as in wild-type seeds. In wild-type seedlings, *HZF* transcription was confirmed to be highly induced in the initial period after heat stress treatment, which suggested once more the regulatory role of HZF. The functional analysis of *HZF* is still in the beginning and further assays are necessary for a conclusive evidence of its particular role in thermotolerance. In order to achieve additional information that would help to functionally characterize *HZF*, transgenic plants were produced for overexpression studies, spatial/temporal expression pattern analysis and subcellular localization.

In summary, the present work contributed to the general knowledge of the mechanisms associated to thermotolerance in plants. Significant advances were achieved through the establishment of a straightforward strategy to identify novel

thermotolerance determinants and a collection of protocols for analysis of their corresponding knockout mutants. The identification of *HZF* and detection of a heat stress-associated phenotype in the *hzf* loss-of-function mutant provided confirmation of the success of the proposed strategy and protocols. As already referred, protein degradation is an important process contributing to plant adaptation to heat stress. Accordingly, HZF putative involvement in the degradation of a specific regulator protein that controls the expression of other genes might be an important mechanism of regulation contributing to thermotolerance.

FUTURE PERSPECTIVES

Even though *P. euphratica* cell suspensions did not presented an exceptional tolerance to elevated temperatures, the natural thermotolerance of this species should be explored. An excellent approach will be the comparison between the transcript profiling of *P. euphratica* and closely related model species subjected to similar heat treatments. The analysis of differential expression will allow the identification of potential candidates mediating heat stress tolerance. The close phylogenetic relationship of *Populus* spp. with *Arabidopsis* will facilitate comparative functional studies and comparative genomics, through the use of a number of tools and techniques currently available to understand gene function in this model system. Also, with the completion of *Populus trichocarpa* sequencing, new tools and genomic resources are rapidly expanding. Similarly to *Arabidopsis*, microarray-based expression data (PopGenExpress) and associated web-based tools (Populus eFP Browser, available at BAR) have been recently developed to enable a community-wide, simple, intuitive representation of poplar transcript abundance data. The use of Populus eFP Browser enables to visualize *Populus* gene expression data during development and after drought treatment, as well as to perform rapid comparisons with putative orthologs from better characterized species such as *Arabidopsis*. These comparisons will enable to test if the homologs may respond to similar stimuli or are involved in the same processes in their respective species. Currently, the data sources displayed in Populus eFP Browser do not include gene expression data relative to heat stress experiments.

Regarding the functional role of HZF in thermotolerance, there are still several questions to be answered. The Southern blot analysis, as well as the complementation and gain-of-function studies that are currently in progress, will be necessary to confirm that the delayed germination observed in heat-treated *hzf* mutant seeds was uniquely due to *HZF* knockout. The use of additional *HZF* insertion alleles is also being considered for phenotype validation. The generated T3 transgenic plants carrying *HZF* promoter::GUS or *HZF ORF-stop::GFP6his* constructs will be used for, respectively, *in situ* gene or protein expression analysis. These assays will provide additional insights concerning the tissues or cells where *HZF* is expressed or the subcellular localization of the corresponding gene product. Also, *in vitro* ubiquitination assays are necessary to determine HZF capacity of E2-dependent protein ubiquitination, therefore confirming its E3 ubiquitin ligase activity. In addition, the interaction protein partners of HZF will be determined for recognizing the proteins that could be susceptible to the HZF control.

The putative redundant function of L-HZF and its E3 ubiquitin ligase activity will also be investigated. Loss-of-function mutants on this gene will be used in the same phenotypic assays as performed for *hzf* mutants. Also, a double knockout mutant will be produced and used in similar phenotypic analysis. These studies will provide, not only evidence of gene redundancy between *HZF* and *L-HZF*, but will also allow to uncover any hidden phenotype at seedling stage. Altogether, these approaches will confirm the proposed model of HZF action on germination, under standard and heat stress-imposed conditions. From these results it is expected to define the role of HZF in controlling the levels of a key regulator of dormancy/germination upon heat stress. Concerning also the validation of the proposed model, further studies are required to investigate the association of HZF to ABA signaling pathway. The determination of HZF and L-HZF protein levels during seed imbibition and germination is necessary to exclude the possibility of protein instability resulting from heat treatment.

Another significant aspect to be further investigated is HZF association to HSF signaling. For evaluating the implication of HZF in regulating heat stress-responsive pathways, the genome-wide gene expression of *hzf* loss-of-function mutants will be analysed under heat stress conditions and compared to equally treated wild-type plants. Also, standard conditions will be considered for comparison. Those genes

differently expressed in mutant and wild-type lines could give a clue for the regulatory pathway in which HZF is involved. It is expected that the lack of *HZF* transcripts would affect the transcript levels of downstream genes, thereby indicating which genes act upstream and downstream of *HZF*. These results will corroborate the hypothesis of HZF being implicated in the regulated degradation of certain targets.