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Review

Some like it hot, some like it warm: Phenotyping to explore thermotolerance diversity

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ABSTRACT

Plants have evolved overlapping but distinct cellular responses to different aspects of high temperature stress. These responses include basal thermotolerance, short- and long-term acquired thermotolerance, and thermotolerance to moderately high temperatures. This 'thermotolerance diversity' means that multiple phenotypic assays are essential for fully describing the functions of genes involved in heat stress responses. A large number of genes with potential roles in heat stress responses have been identified using genetic screens and genome wide expression studies. We examine the range of phenotypic assays that have been used to characterize thermotolerance phenotypes in both Arabidopsis and crop plants. Three major variables differentiate thermotolerance assays: (1) the heat stress regime used, (2) the developmental stage of the plants being studied, and (3) the actual phenotype which is scored. Consideration of these variables will be essential for deepening our understanding of the molecular genetics of plant thermotolerance.

Contents

1. Introduction

Plants are sessile organisms which constantly experience changes in their environments. Some of these changes are stressful; they are detrimental to plant growth and development. Elevated temperatures result in complex and poorly understood effects on plant phenology [1] and cause plant heat stress. Heat stress significantly affects cellular homeostasis including both protein and membrane stability. To avoid or minimize the detrimental effect of heat stress, plants must respond appropriately to the challenges of stressful elevated temperatures. Much effort has been invested in understanding plant heat stress responses, efforts which have recently been motivated by concerns about potential decreases in crop productivity caused by global warming. It is optimistically believed with some justification that a better understanding of the mechanisms underlying plant heat stress responses may facilitate the development of technologies and breeding strategies for improving crop thermotolerance.

Abbreviations: HSP, heat-shock protein; BT, basal thermotolerance; SAT, short-term acquired thermotolerance; LAT, long-term acquired thermotolerance; TMHT, thermotolerance to moderately high temperatures.

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A large number of genes that are induced or repressed by heat treatment have been identified by transcriptome profiling using microarrays [2–10]. Demonstrating the functions of heat stress response genes using molecular genetic tools has become a bottleneck because of the large numbers of genes that have been identified. Isolating or generating genetic variants of target genes is time consuming and is not always fruitful as reverse genetic approaches often do not result in altered heat stress response phenotypes. For instance, in a study of 48 T-DNA insertions in Arabidopsis genes implicated in heat stress response based on their expression profiles, only one gene had an acquired thermotolerance phenotype [11]. Although the function of some heat stress response genes may be obscured due to genetic redundancy, recent studies show that in many cases uncovering heat stress response phenotypes depends on choosing appropriate heat stress phenotype assays.

The difficulty in selecting appropriate phenotypes for characterization may have resulted in an underestimation of the complexity of the heat stress response in plants, since heat stress treatments performed in a laboratory are often simple and may not reflect 'real world' heat stress conditions. A number of environmental factors including, but not limited to, ambient air temperature and light intensity create a range of heat stress conditions that plants may experience during their lifetime [12]. These heat stress conditions can threaten the fitness and productivity of plants in combination with other stress factors, such as water limitation and high UV irradiation. Plants have evolved a complex heat stress response system to cope with these heat stress conditions. It is made up of overlapping subsets of genes required for thermotolerance in response to specific environmental conditions [13–16]. We will refer to these multiple kinds of responses as 'thermotolerance diversity'.

The concept of thermotolerance diversity suggests that phenotyping with only a few simplified thermotolerance assays may not be adequate for elucidating the functions of the large number of potential heat stress response genes identified in genomic studies. Instead a systematic phenotyping approach that includes a range of heat stress conditions may increase the chances of identifying the functions of potential heat stress response genes. Heat stress phenotyping can be performed at different temperatures, for various amounts of time, and with a range of heating devices, each of which has its own advantages and disadvantages. Choosing an appropriate phenotype to measure is critical because the function of a heat stress response gene may contribute to thermotolerance differentially across tissues and growth stages. Because these issues have not been discussed in detail in the plant heat stress response literature, we thought that it would be worthwhile to highlight and summarize these issues.

We summarize and discuss the phenotyping methods used in plant molecular genetic studies of heat stress response. To underscore the importance of phenotyping, we have not included an in depth discussion of the biological roles of heat stress response genes or signaling networks, which have been extensively reviewed elsewhere [17–24]. Instead we focus on critical parameters used in various thermotolerance assays. We also do not attempt to review the literature detailing phenological studies on temperature effects on crop yields, which is largely separate from the literature describing molecular genetic approaches to these questions. By focusing on the parameters used in thermotolerance assays we hope to provide a useful framework for designing future studies on plant heat stress response. This review is divided into two major sections; one focuses on the model plant Arabidopsis (*Arabidopsis thaliana*) in which many important molecular genetic discoveries have been made. The second section focuses on heat stress phenotyping in crop plants. Finally we briefly discuss related issues in emerging model plant functional genomics systems.

2. Phenotyping methods for studying heat stress response in Arabidopsis

Arabidopsis is the most widely used species for plant molecular genetics. The history and advantages of its use as a model system for molecular genetics has been comprehensively described [25] and this 'simple plant' has been extensively used to study the complex heat stress response in plants [18]. Knowledge gained from Arabidopsis research serves as a reference point for work in other plant species including economically important crops.

Organismal thermotolerance is the most widely used phenotype for describing the biological functions of heat stress response genes. Thermotolerance functions of a heat stress response gene were first described in an Arabidopsis study using transgenic plants overexpressing HSFA1A (or HSF1, a transcription factor) and HSFA1A/GUS fusion proteins [26]. The heat stress regimes and heating device used for determining basal and acquired thermotolerance levels were clearly described. This study showed a dramatic difference in the viability of wild-type *versus* transgenic seedlings after the heat stress treatments. In our survey of the field, viability is the most frequently reported output trait used to describe thermotolerance in studies using Arabidopsis seedlings (Table 1).

The size of Arabidopsis seedling is small enough so that a large number of seedlings can be grown and heat-treated in a single petri dish, which increases the throughput of experiments. Additional output traits such as hypocotyl elongation and chlorophyll accumulation have also been successfully employed to efficiently identify mutagen-induced Arabidopsis mutants with thermotolerance defects [27,28]. New heat stress regimes were also adopted to study heat stress-related genes in both forward and reverse genetic studies [29–31]. In the following sections we summarize and discuss three major parameters that need to be considered for heat stress response phenotyping: the heat stress regime, the developmental stage of the plant to be studied, and the thermotolerance-associated output trait. The last two parameters are often considered simultaneously so we will discuss them in one section.

2.1. Selection of heat stress regime and heating device

The heat stress regime used in an experiment is critical for successfully identifying thermotolerance phenotypes. Several distinct heat stress regimes have been developed for characterizing the phenotypes of Arabidopsis T-DNA knockout (KO) mutants. These regimes have uncovered functional specificities of heat stress response genes in different types of thermotolerance [11,14–16,30]. Four major thermotolerance types can be categorized based on the heat stress regimes used in these studies: basal thermotolerance (BT), short-term acquired thermotolerance (SAT), long-term acquired thermotolerance (LAT), and thermotolerance to moderately high temperatures (TMHT; Table 1). The heat stress regimes used to assay these types of thermotolerance are shown schematically in Fig. 1A. Minor differences in temperature and treatment duration are found in different studies, presumably due to differences between the heating devices, the genetic backgrounds, and the growth conditions used in each experiment.

Three types of heating device have been employed in Arabidopsis thermotolerance studies: heating blocks, water baths, and growth chambers/ovens (Table 1). For seedlings grown in petri dishes water baths seem to be the most efficient of the three to deliver heat stress temperatures. It takes less than 45 min at 44 °C to kill non-acclimated wild-type Col-0 seedlings in a sealed petri dish submerged in water bath, while more than 1 h at 45 °C is required on heating block or in growth chamber to do the same (Table 1). This difference is presumably due to the better thermal conductivity of water than air.

Table 1
The major parameters for thermotolerance phenotyping of Arabidopsis mutant or transgenic lines.

Mutant or transgenics[a]	Stage/tissue	Output trait	Heat stress regime[b]				Heating device[c]	Ref.
			BT	SAT	LAT	TMHT		
ACS7	Seedling	Cotyledon chlorosis	↓[d]				GC	[90]
ATJ1 (*AtDjB1*)	Seedling	Viability	↓				NC	[46]
		TTC[e] reduction	↓					
		Electrolyte leakage	↓					
		Lipid peroxidation	↓					
ATJ2/ATJ3 (KO)	Seedling	Viability	↓				GC	[91]
ATJ2 or *ATJ3* (OE)			↑					
BAX INHIBITOR-1	Seedling	Electrolyte leakage	↓				IO	[92]
	Adult plant	Viability	↓					
BOB1	Seed	Germination	↓				WB	[93]
	Seedling	Viability	↓	↓				
bZIP28	Seedling	Viability	↓				GC	[94]
CAM3 (KO)	Seedling	Viability	↓				NC	[95]
		Lipid peroxidation	↓					
(OE)		Viability	↑					
		Lipid peroxidation	↑					
CHIP (OE)	Adult plant	Seed production	↓				GC	[96]
cpHSC70-1	Seed	Root growth after germination	↓				WB	[97]
	Seedling	Viability			×			
		Chlorophyll content			↓			
CPR5	Seedling aerial part	Electrolyte leakage		×		↑	GC	[33]
COI1, *NPR1*				×		↓		
CTL1 (*HOT2*)	Etiolated seedling	Hypocotyl elongation		↓			NC	[40,98]
	Seedling	Viability		↓				
DGD1	Seed	Germination	×				GC	[14]
	Etiolated seedling	Chlorophyll accumulation		↓		↓		
		Hypocotyl elongation		×				
	Seedling	Viability		↓				
DREB2A (CA)[f]	Seedling	Viability	↑				IO	[99]
(KO)				↓				
DREB2C (OE)	Seedling	Viability	↑				GC	[100]
FAD3/FAD7/FAD8	Leaf	Photosynthetic functions	↑				GC	[101]
	Seedling	Quantum yield of PSII				↓		
		Fresh weight				↓		
		Viability				↓		
FAD7/FAD8	Seedling	Viability				↑	GC	[102]
	Leaf	O_2 evolution	↑					
FES1A	Seedling	Viability		↓			GC	[37]
FTSH11	Seed	Germination	↓				GC	[32]
	Etiolated seedling	Chlorophyll accumulation		↓		↓		
		Hypocotyl elongation		×				
	Seedling	Viability		↓				
	Leaf	Chl a/b, quantum yield of PSII				↓		
	Reproductive stage	Seed production				↓		
GASA5 (KO)	Seedling	Viability	↑				NC	[103]
(OE)			↓					
GSNOR (*HOT5*)	Etiolated seedling	Hypocotyl elongation		↓			NC	[104]
	Leaf disc	Chlorophyll content		↓				
HSA32	Etiolated seedling	Hypocotyl elongation	↓	×	↓		WB, GC	[11,15,30]
	Seedling	Viability	↓	×	↓	×		
HSBP (KO)	Seed	Germination	×				WB	[42]
	Etiolated seedling	Hypocotyl elongation	×	×				
	Seedling	Viability		↑				
(OE)	Seedling	Viability		↓				
HSFA1A/B/D	Seed	Germination	↓				WB, GC	[9,10]
	Seedling	Viability	↓	↓	↓	↓		
HSFA1A-GUS, *HSFA1B* *HSFA1B-GUS* (OE)	Seedling	Viability	↑				HB	[26,105]

Table 1 (*Continued*)

Mutant or transgenics[a]	Stage/tissue	Output trait	Heat stress regime[b]				Heating device[c]	Ref.
			BT	SAT	LAT	TMHT		
	Etiolated seedling	Hypocotyl elongation		×	↓		WB	
HSFA2 (KO)	Seedling	Viability		×	↓			[11,106]
(OE)	Seedling	Viability	↑	↑			GC	
	Seed	Germination	↓					
HSFA3 (KO)	Etiolated seedling	Hypocotyl elongation		↓			IO	[86,87]
	Seedling	Viability	↓	↓				
(OE)	Seedling	Viability	↑					
HSFA7A[g]	Seedling	Viability		↓			GC	[31]
HSFB1/HSFB2B	Seedling	Viability	↑	↓	↓		WB	[89]
	Seed	Germination and growth	↓					
HSP101 (*HOT1*)	Etiolated seedling	Hypocotyl elongation	↓	↓			WB,	[15,28,39]
	Seedling	Viability	↓	↓	↓	×	GC	
HSP70	Seedling							
	Detached leaf	Viability	×	↓		HB		[107]
	Seedling	Relative growth rate				↑	GC	
ISPS	Adult plant	Viability	↑				IO	[49,108]
MBF1C (KO)	Seedling	Viability	↓	×			NC	[109]
(OE)			↑					
MGE2	Seedling	Viability	×	×	×	↓	WB, GC	[15]
MSH1/RECA3	Adult plant	Viability				↑	GC	[110]
NOA1 (*RIF1*)	Seedling	Viability	↓				NC	[111]
		Viability		↓				
P5CS1 (heat inducible)	Seedling	Electrolyte leakage		↓			NC	[112]
		Lipid peroxidation		↓				
PLC9 (KO)	Seedling	Viability	↓	↓			NC	[113]
(OE)			↑	↑				
PP5 (KO)	Seedling	Viability		↓		↓	WB,	[114]
(OE)			↑			↑	GC	
PP7 (KO)	Seedling	Viability	↓				NC	[115]
(OE)			↑					
	Seed	Germination				↑		
RCA1 (thermostable)	Adult	Photosynthesis				↑	GC	[116]
	Reproductive stage	Seed production				↑		
ROF1	Seedling	Viability		×	↓		IO, WB	[34]
ROF2	Seedling	Viability		×	↑		IO, WB	[35]
RPN1A	Seed	Hypocotyl length	↓				NC	[117]
RPS1	Seedling	Viability		↓				[118]
	Adult plant	Viability				↓		
SGT1A	Seedling	Viability		↓			GC	[119]
sHSP-CIs	Etiolated seedling	Hypocotyl elongation		↓			NC	[84]
	Seed	Germination	↓					
SIZ1	Etiolated seedling	Hypocotyl elongation		×			GC	[120]
	Seedling	Viability	↓[h]	×				
SUMO1 (OE)	Seedling	Viability			↓		NC	[121]
tAPX	Seedling	Viability	↑				NC	[122]
		Root length				↑		
	Seed	Germination	×					
TIL1	Etiolated seedling	Hypocotyl elongation	×	×	×		WB	[41]
	Seedling	Electrolyte leakage		×				
		Viability	↓	↓				
	Seed	Cotyledon greening				↓		
TMS1	Pollen	Fertility, germination				↓	NC	[123]
	Seed	Germination	↓					
UVH3[g]	Etiolated seedling	Hypocotyl elongation		↓			IO	[13]
	Seedling	Viability, lipid peroxidation		×				
		Root growth						

Table 1 (*Continued*)

Mutant or transgenics[a]	Stage/tissue	Output trait	Heat stress regime[b]				Heating device[c]	Ref.
			BT	SAT	LAT	TMHT		
VPS53 (*HIT1*)	Seed	Germination				↓	GC	[29,48,124]
	Seedling	Viability	×	×		↓		
		Electrolyte leakage				↓		
		Lipid peroxidation				×		
WRKY25, 26, 33	Seedling	Viability	↓				GC	[125]
XPO1A (*HIT2*)	Seedling	Viability	↓	×	×	↓	WB	[16]

[a] The genes correspond to the completely or partially loss-of-function mutant/transgenic lines caused by T-DNA insertion (KO), mutagen-induced mutation, or RNA interference if not specified. OE indicates the overexpression of specified transgenes. The gene names are shown according to The Arabidopsis Information Resource (TAIR).
[b] BT, basal thermotolerance; SAT, short-term acquired thermotolerance; LAT, long-term acquired thermotolerance; TMHT, thermotolerance to moderately high temperatures.
[c] GC, growth chamber/climate chamber; HB, heating block; IO, incubator/oven; NC, not clear; WB, water bath.
[d] Increase (↑), decrease (↓), or no change (×) in thermotolerance as compared to the wild-type output trait under each HS regime.
[e] Triphenyltetrazolium chloride.
[f] Constitutively active.
[g] The representing gene in studies involving more than 10 genes.
[h] This BT test was done at 39 °C for 4 h.

Water baths are appropriate for short-term high temperature treatments performed in the dark to avoid the complex effect of phototoxicity. If heat stress treatment in the light is needed, such as in TMHT, a growth chamber is the most commonly used approach for delivering a heat stress. Maintaining temperature stability temporally and spatially in a growth chamber is a significant problem for generating reproducible results. Caution in preventing uneven heating in growth chambers must be used to avoid heat stress gradients across and between plates. The door of the chamber should be kept closed as much as possible and petri dishes should be placed directly on a large pre-equilibrated heat sink such as a block of metal. Although a heating block with an appropriate light source can also be used this approach, it has not commonly been used for heating petri dishes in heat stress response studies.

BT is the ability of a plant to tolerate heat stress, generally 44–45 °C for Arabidopsis seedlings, without acclimation, a prior exposure to moderately high temperatures. BT has been measured at temperatures as low as 30 °C with durations up to 5 d [32] or 38 °C for 16 h [33]. Heat acclimation at moderately high temperature induces the synthesis of many heat shock proteins (HSPs) and leads to enhanced tolerance to severely high temperature, which is named 'acquired thermotolerance'. Prolonged exposure to moderately high temperatures induces some HSPs and thermotolerance against these conditions is probably different from BT or acquired thermotolerance. Instead, we proposed that this type of thermotolerance needs to be separately classified as TMHT, as it is distinct from acquired thermotolerance associated with a challenge at severely high temperature. This difference is nicely illustrated by a recent study on a mitochondrial co-chaperone in the DnaK/HSP70 complex, MGE2. MGE2 is required for tolerance to prolonged exposure to 35 °C for up to 9 d, but not for either basal or acquired thermotolerance associated with acute heat stress conditions at 44 °C. By contrast, HSPs that are involved in basal and acquired thermotolerance such as HSP101 play a minimal role in TMHT [15]. These data indicate a mechanistic difference between the thermotolerance required for survival under moderate and severe heat stress conditions. Although these modes of thermotolerance appear to be experimentally separable, there is evidence for at least a limited overlap in the plant response mechanisms. A genetic screen designed to identify genes involved in TMHT resulted in the isolation of the *heat-intolerant* (*hit*) mutants [29]. *HIT2* encodes a nuclear export receptor XPO1A that is required for BT and TMHT but not for acquired thermotolerance [16]. This observation suggests that some heat stress response genes are involved in tolerance both to chronic and certain mode of acute heat stress.

The recent identification and characterization of the heat stress-associated 32-kDa protein (HSA32) expands the assays for acquired thermotolerance phenotypes that need to be considered when thinking about plant heat stress response [30]. HSA32 is required for LAT, a term which first appeared in [34]. The heat stress regime for LAT includes a long recovery period (48–72 h) between the acclimation treatment and the high temperature challenge. This is in contrast to the short recovery time (usually less than 2 h) in the heat stress regime used for acquired thermotolerance assay in many studies (Fig. 1A). To distinguish LAT from the acquired thermotolerance associated with a short recovery time, we have renamed the latter SAT [15]. The heat stress challenge in a LAT regime is less severe than for SAT (Fig. 1) because acquired thermotolerance gradually decays after a long recovery [30]. The LAT heat stress regime that revealed the mutant phenotype of *HSA32* T-DNA KO plant is also effective in identifying LAT phenotypes in KO mutants of *HSFA2* [11], *ROF1* [34], and *ROF2* [35]. LAT has only been reported in studies using Arabidopsis. It remains to be demonstrated that this type of thermotolerance exists in other species although this seems likely since the genes involved in LAT responses are conserved among land plants [36].

Another variable in heat stress regimes is how acclimation is performed. Gradual acclimation by continuously increasing temperature from 22 °C to 45 °C over a period of 6 h (Fig. 1B) leads to higher thermotolerance levels and higher expression of heat stress response genes than a typical step-wise sudden acclimation [31]. The presence or absence of a recovery period between acclimation and heat stress challenge must also be considered [37]. A recovery period at non-stress temperature after acclimation treatment results in higher thermotolerance levels, probably due to sufficient time elapsing allowing for HSP accumulation [37]. It is to be seen whether these heat stress regimes are associated with novel types of thermotolerance.

The functional specificities of heat stress response genes in different types of thermotolerance cannot simply be predicted by transcript profiles. Instead, a better correlation between phenotype and protein levels was shown in some cases [15,30]. It may be helpful to first analyze the protein profiles of genes of interest to determine the kinds of heat stress regimes needed to uncover heat stress response phenotypes.

2.2. Selection of growth stage and output traits

Arabidopsis has distinct developmental stages that should be assayed separately for thermotolerance phenotypes [38]. Thermotolerance phenotypes of seeds, seedlings, adult plants, reproductive

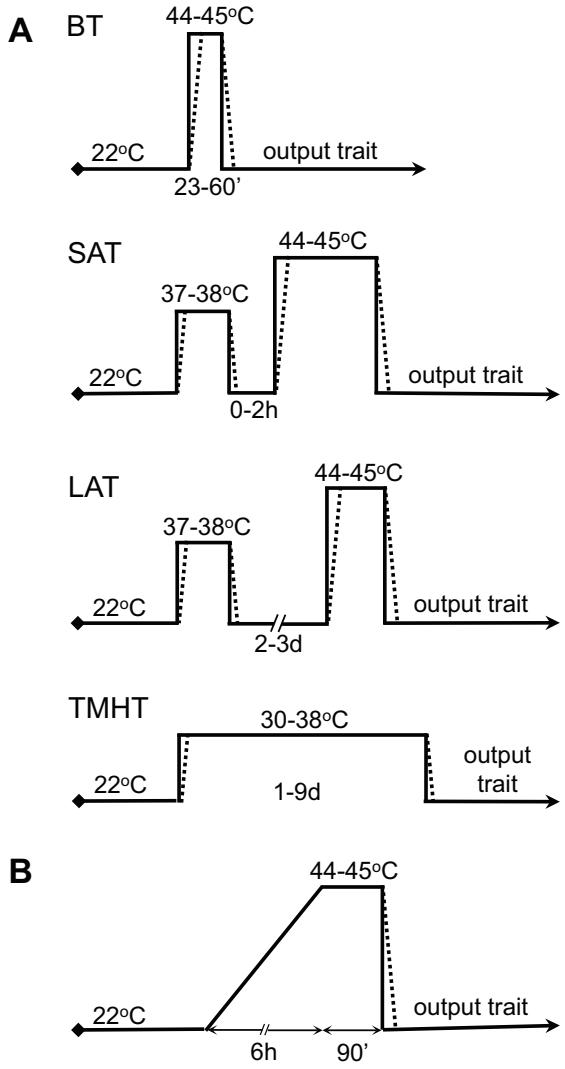

Fig. 1. Heat stress regimes used for thermotolerance phenotyping in Arabidopsis. (A) Schemes of heat stress regimes for four major types of thermotolerance: basal thermotolerance (BT), short-term acquired thermotolerance (SAT), long-term acquired thermotolerance (LAT), and thermotolerance to moderately high temperatures (TMHT). The schemes show temperatures that are often used in young seedlings. The temperature and duration of treatment varies depending on the exact tissue tested or experimental design used. The dashed lines indicate the time-lag between the applied temperature and the sample temperature, which vary with the heating device used. The output traits are assessed after various periods of incubation after the heat treatment. (B) The heat stress regime for gradient acclimation is adapted from [31].

organs, and detached leaves have been determined in various experiments (Table 1). Since tissues assayed at different growth stages have varied levels of thermotolerance [13,39,40] heat stress regimes have to be carefully designed to account for plant age. When characterizing the thermotolerance phenotypes of either a new mutation or a transgene the possibility of developmental phenotypes should be carefully evaluated to avoid confusing the thermotolerance effects due to altered growth with true changes in cellular thermotolerance. The extended recovery period before the final, severe, heat stress in LAT assays provides a long window for changes in development to affect BT, so special caution should be taken in these experiments to rule out developmental effects. One also needs to consider whether and to what extent any acclimation treatment may differentially retard growth among genotypes. Finally, in order to reveal the effect of acclimation any heat stress challenge measuring SAT or LAT must be severe enough to overcome BT at any relevant stage of development.

Arabidopsis seeds, which contain high levels of HSPs, can tolerate more than 2 h at 44–45 °C after 3-d of imbibition at 4 °C without acclimation, which is considered as BT. The rate and extent of germination are easy and reliable phenotypes that can be assessed after the heat treatment. To our knowledge no report has been published on the thermotolerance of dry Arabidopsis seeds. It would be of interest to see whether dry seeds can be used for assessing thermotolerance.

Young seedlings, either dark- or light-grown, are the most frequently used developmental stages as they show more phenotypes than the simple germination phenotype observed in seeds. Viability of seedlings is the most popular phenotype for thermotolerance assays (Table 1). Viability is usually scored as the ability of the seedlings to generate new green leaves under permissive temperatures after a heat stress treatment. Hypocotyl and root elongation can be measured instead of the emergence of green leaves and have the advantage of being quantitative. Seedlings are grown in the dark before and after heat treatment to induce etiolation for hypocotyl elongation assays [28]. As with viability, this output trait can be used under the heat stress regimes for BT, SAT, and LAT. However, not all heat stress response mutants that show defect in viability are also defective in hypocotyl elongation (Table 1). For example, *HSP101* and *HSA32* KO mutants are severely defective in hypocotyl elongation [28,30], but mutants of *DGD1* [14], *FTSH11* [32], *TIL1* [41], *HSBP* [42], and *BOB1* (Kaplinsky and Perez, unpublished data) are not. In contrast the *uvh3-1* mutant is defective in hypocotyl elongation but not in viability [13]. Using either one of these traits as the only phenotype for thermotolerance screening is not sufficient for identifying many important heat stress response genes.

Accumulation of chlorophyll in cotyledons following acclimation of heat stress challenged dark-grown seedlings and subsequent exposure to light is another useful phenotype for identification of genes with functions in thermotolerance [14,27,32]. The two thermotolerance-conferring proteins, DGD1 and FTSH11, identified by this method are localized in chloroplasts and are required for normal function of the organelle at high temperatures. In contrast, the loss-of-function mutant of *HSP101*, *hot1-1*, is not defective in chlorophyll accumulation after heat stress [14,32]. These observations illustrate the importance of considering different plant needs for heat stress response genes functioning in different subcellular compartments.

Yellowing or bleaching of cotyledons or true leaves is a visible phenotype for light-grown seedlings, which can be quantified by measuring chlorophyll content, Chl a/b ratio, and the quantum yield of photosystem II (PSII; Table 1). Measurement of quantum yield of PSII is advantageous as this method is nondestructive and mutants identified using this approach can subsequently be used for other purposes including setting up crosses.

It is more challenging to use adult Arabidopsis plants for thermotolerance phenotyping than young seedlings. Unlike young seedlings that can be grown in large numbers in petri dishes, adult plants grown in pots are larger and growth chambers must usually be used for thermotolerance assays. Concerns about this type of heating device have been mentioned in previous sections. Nevertheless, some important genetic components of thermotolerance with distinct phenotypes in adult plants have been successfully revealed (Table 1). Detached organs, most often leaves, are alternative choices for thermotolerance phenotyping in mature plants and are easier to use than whole plants. Detached leaves or leaf discs incubated in a petri dish can be heated in a manner similar to seedling treatments. Several output traits can be measured on the detached leaves after heat stress treatment including chlorophyll content, electrolyte leakage, and electrochromic absorbance (see below).

Plant reproductive organs are far more sensitive to heat than other tissues [43,44], and several studies on reproductive organ thermotolerance have been reported in Arabidopsis (Table 1). Unlike assays used for seeds and seedlings, which can be performed within one or two weeks of germination, assaying the heat tolerance of reproductive organs requires much longer periods of plant growth. While this can limit the scale of experiments, the reproductive stage of plant development is obviously relevant to plant productivity and is a major concern in agriculture, so studies of gene function at this stage are of great interest.

Some output traits of thermotolerance assays depend on the growth stage such as hypocotyl elongation in dark-grown seedlings. Other traits can be used at many different growth stages or in detached organs. For example, lipid peroxidation measured using thiobarbituric acid-reactive substances assays and electrolyte leakage are frequently employed on seedlings as well as tissues collected from adult plants (Table 1). A triphenyltetrazolium chloride reduction assay has been used to measure root vitality and could presumably be used on other organs [45,46]. The limitations of these methods have been discussed in [27]. Recently, three biophysical methods, circular dichroism (CD) spectroscopy, electrochromic absorbance transients, and thermoluminescence, have been employed to assess the effect of isoprene on the thermal stability of thylakoid membranes in transgenic Arabidopsis [47]. Arabidopsis does not naturally emit isoprene and in this study transgenic Arabidopsis plants constitutively expressing an isoprene synthase gene from poplar produced isoprene and acquired enhanced tolerance to heat stress treatments. CD spectroscopy determines structural changes in the thylakoid membranes. Flash-induced electrochromic absorbance change at 515 nm is used to monitor perturbation in ion permeability of membranes. Thermoluminescence detects the structural alterations in both the donor and acceptor side of PSII (refer to [47] for details and references to these techniques). Viability and growth rate thermotolerance phenotypes under BT and TMHT heat stress regimes are in good agreement with the indexes determined by these biophysical measurements, suggesting that they may be of general use in heat stress phenotyping.

Different output traits are assayed after various periods of recovery following heat stress treatments (Fig. 1). Traits such as viability, lipid peroxidation, and hypocotyl elongation require several days for measurable phenotypes to develop. Other output traits such as photosynthetic efficiency and electrolyte leakage are commonly, but not always, assayed immediately after the heat treatment. For instance, some studies report cellular electrolyte leakage after 2–3 d of incubation at normal condition following heat stress treatment. This extended recovery time allows investigation of secondary effects of cell death as opposed to primary events. It should be noted that there is no linear relationship between early electrolyte leakage event and viability [41]. Lipid peroxidation levels are also not correlated well with the survival rate after heat stress treatment [48]. Because of this temporal complexity continuous monitoring of output traits during or after stress treatment may be needed in some cases as phenotypes might only be revealed within a short window of time [49].

3. Phenotyping methods for studying heat stress response in crop plants

By the end of this century growing season temperatures in the tropics and subtropics are predicted to exceed the hottest seasonal temperatures recorded in the last century [50]. As many crops will experience warmer environments, which are predicted to reduce productivity, climate change raises significant concerns about food security. Although it can be less convenient to study the genetic basis of heat stress tolerance in crop plants than in Arabidopsis the information gained from non-model systems has unique value as these studies can have direct implications for agriculture. Mechanisms of heat stress tolerance which exist in important crop species may not exist in Arabidopsis, for example the emission of isoprene mentioned above [51]. Increasing environmental temperatures often accompany other environmental stresses such as drought, high irradiance, and disease in field situations [52,53]. Thus, appropriate phenotyping of responses to heat stress coupled with other stresses is important to the study of heat tolerance of crop plants. Although there are limitations when working in crop plants there is a large literature focused on heat stress response and thermotolerance in crop plants.

High temperatures constrain plant growth and can adversely affect seed germination, photosynthetic efficiency and other core metabolic processes, pollen viability, respiration, water relations, protein and membrane stability. Because of the wide range of effects that high temperatures have on plants many thermotolerance assays have been employed to assess the effects of a multitude of factors on heat stress tolerance of various plant species and in the identification of heat tolerant germplasms by plant breeders. For plant breeders an important objective is the development of an effective set of thermotolerance markers for marker assisted selection (MAS).

The three major parameters used in studies that compare thermotolerance levels in different crop cultivars, mutant *versus* wild type plants, and transgenic *versus* non-transgenic plant are summarized in Table 2. In the following sections we discuss these parameters.

3.1. Selection of heat stress regime and heating device

BT, SAT, and TMHT but not LAT assays have been reported in studies using crop plant species (Table 2). For consistency we will use the same definitions of thermotolerance types described in Section 2.1.

There is only one report in crop plants detailing the characterization of a heat stress response gene genetic knockout: maize plants lacking HSP101 are defective in both BT and SAT [54]. Transgenic approaches have however been used extensively to assess the role of heat stress response genes in crop plant thermotolerance. One example is the constitutive expression of HSFA1A in tomato which enhances SAT in seedlings. Down-regulation of the same gene by co-suppression decreased the BT and SAT of seedlings and the TMHT of mature green fruit [55]. Other examples in which overexpression of single HSPs or metabolic enzymes enhanced one or multiple types of thermotolerance are summarized in Table 2.

Assays for BT, SAT, and TMHT have also been used to differentiate heat-tolerant and -sensitive cultivars of lima bean, peanut, potato, rice, sunflower, and wheat. The genetic basis underlying heat-tolerant phenotypes in these species have yet to be identified. In general these kinds of studies do not investigate whether heat-tolerant lines have increases in multiple types of thermotolerance relative to heat-sensitive lines.

Although crop plants are larger than Arabidopsis, the heating devices used for crop plants are similar to those used in Arabidopsis research because many crop plant assays are conducted on seedlings or small portions of plants such as leaves or leaf punches. Thermotolerance studies on mature crop plants are either performed in large growth chambers or greenhouses.

3.2. Selection of growth stage and output trait

The temperature and duration of heat stress treatments resulting in changes in growth and development vary between plant

Table 2
The major parameters for thermotolerance phenotyping of crop plants.

Plant	Line[a]	Stage/tissue	Output trait	Heat stress regime		Heating device[b]	Ref.
Carrot	*HSP17.7* (OE, KD)	Cell culture, Detached leaf	Growth, electrolyte leakage	BT SAT	50 °C, 1–3 h 37 °C, 2 h → 23 °C, 1.5 h → 48 °C, 30 min	WB	[126]
Chrysanthemum	*DREB1A* (OE)	Seedling	Viability, electrolyte leakage	BT	45 °C, 36 h	GC	[127]
Cotton	Cultivars	Seedling	Chlorophyll accumulation	BT SAT	50 °C, 30 min 40 °C, 2 h → 50 °C, 30 min	GC	[67]
		Leaf	Electrolyte leakage Photosynthetic functions	TMHT	38 °C/20 °C (12/12 h), 7 d 46 °C/30 °C day/night	GC	[57,64]
		Seed	Ripening			GH	[128]
Creeping bendgrass	*SAG12-ipt* *HSP18-ipt*	Adult plant	Root growth Chlorophyll content	TMHT	35 °C, 7–14 d	GC	[129,130]
Lima bean	Cultivars	Leaf disc	Electrolyte leakage	SAT	37 °C, 0–24 h → 45 °C, 30–120 min	WB	[131]
Maize	*HSP101* (KO)	Seedling	Shoot and root length	BT SAT	48–50 °C, 1 h 40 °C, 1 h → 28 °C, 1 h → 48 or 50 °C 1 h	WB	[54]
Peanut	Cultivars	Leaf disc	Chlorophyll accumulation	SAT	38 °C, 4 h → 50 °C, 30 min	HB	[132]
Potato	Cultivars	Leaf disc	Electrolyte leakage	BT	47 °C, 4 h	WB	[133]
Rice	*AtHSP101* (OE)	Seedling	Viability	BT	45 °C, 3 h; 47 °C, 2 h; 50 °C, 40 min	IO	[134]
	sHSP17.7 (OE)	Seedling	Viability, electrolyte leakage	BT	50 °C, 2 h	NC	[135]
	SBPase (OE)	Seedling	Growth, CO_2 assimilation	TMHT/BT	35–45 °C, 2 h	GC	[136]
	Cultivars	Spike Adult plant	Grain filling duration Grain filled, flag leaf size, plant height, grain number, panicle length	TMHT	38 °C, 6 h 38 °C, 48 h	GC GH	[70,137]

Table 2 (*Continued*)

Plant	Line[a]	Stage/tissue	Output trait	Heat stress regime		Heating device[b]	Ref.
Sunflower	Cultivars and hybrids	Seedling	Viability	BT SAT	51 °C, 1–2 h 28 to 42 °C in 2.5 h → 42 °C, 1 h → 49 or 51 °C 2 h	IO	[138]
Tobacco	Bacterial *PanD* (OE)	Seed Seedling	Germination Growth	TMHT	42 °C, 12 d 35 °C, 7 d	GC	[139]
	BhHSF1 (OE)	Seedling	Viability	BT SAT	48 °C, 2.5 h 40 °C, 3 h → 50 °C, 3 h	WB	[140]
	FAD7 (KD)	Seedling	O_2 evolution Viability	BT BT TMHT	40–45 °C, 5 min 47 °C, 2–3 d 36 °C, 60 d	GC	[102]
	Rice *HSP101* (OE)	Seedling	Viability	BT	49 °C, 70 min	WB	[141]
	Spinach *BADH* (OE)	Adult plant	Relative growth in dry weight Photosynthetic functions	BT TMHT/BT	40–50 °C, 4 h 30–45 °C, 2 h	GC	[142,143]
	Sweet pepper *GPAT* (OE)	Adult plant	Net photosynthetic rate, *Fv*/*Fm* and photochemical efficiency (Φ_{PSII}) of photosystem II	TMHT/BT	35–48 °C, 4 h or during recovery after 45 °C, 8 h	NC	[144]
Tobacco	Tomato *MT-sHSP* (OE, KD)	Seedling	Viability	BT	46–48 °C, 2 h	GC	[145]
Tomato	Bacterial *CodA* (OE)	Seed	Germination	BT TMHT	40–55 °C, 90 min 34 °C or 40/30 °C (12/12 h), 12 d	GC	[146]
	Chloroplast *LeHSP100/CLPB* (KD)	Seedling 6-wk-old plant	Fresh weight, plant height Viability *Fv*/*Fm*	TMHT SAT	34 °C, 7 d 38 °C, 2 h → 46 °C, 2 h 2 h each at 38, 40, 42, 44, 46 °C	GC	[147]
	HSFA1A (OE, KD)	Seedling Fruit	Viability Ripening	BT SAT TMHT	45 °C, 1 h 45 °C, 1 h → 51 °C, 1 h 42 °C, 2 d	GC	[55]
Wheat	Cultivars	Leaf segment	TTC[c] reduction, electrolyte leakage	BT SAT	50–52 °C, 10–60 min 37 °C, 17 h → 50 °C, 10–60 min 39 °C, 48 h → 49 °C, 30 min	WB	[61,63,148]
	Cultivars	Spike	Grain filling duration Kernel weight	TMHT	30/25 °C (16/8 h), 3 d 35/30 °C (14/10 h), 3 d	GC	[68,69]
	Ditelosomic lines	Leaf segment	Chlorophyll accumulation	SAT	40 °C, 4 h → 48 °C, 30 min	HB	[149]

[a] Mutant or transgenic lines of indicated genes; heat-tolerance or -sensitive cultivars; KO, knockout; KD, kockdown; OE, overexpression.
[b] GC, growth chamber/climate chamber; GH, green house; HB, heating block; IO, incubator/oven; NC, not clear; WB, water bath.
[c] Triphenyltetrazolium chloride.

tissues and growth stages. Seeds, seedlings, mature leaves, panicles or spikes, and fruits have all been used in crop thermotolerance studies (Table 2).

The germination of heat-treated seeds is commonly assayed by scoring radicle emergence for thermotolerance phenotyping of crop plants seeds. It has been used to measure both BT and TMHT (Table 2). Seed maturity and pre-treatments such as scarification, cold or warm stratification, and dry storage must be carefully considered prior to analyzing BT of seeds as changes in any of these may affect thermotolerance [56,57]. As an example of the importance of these parameters, it has been suggested that seed traits including seed weight, volume, and density can be used to differentiate relative heat tolerance in cultivars of upland cotton [57,58]. Viability is the most commonly used phenotype in seedling studies, while growth rates determined by measuring fresh or dry weight and shoot and root length after heat stress treatment allows for quantitative measurements of thermotolerance.

Reproductive phases of plant development tend to be far more sensitive to heat stress than earlier stages [43]. Brief periods of heat stress can significantly suppress fertility in many species. Determining the thermotolerance of reproductive organs of cereal plants is of great interest as thermotolerance at this stage is directly related to grain filling and plant yield in heat stress conditions. Both anthesis and grain filling of many cereal crops grown in temperate regions are influenced by atmospheric temperature fluctuations [59]. Spikelet fertility and grain filling have been compared in heat-tolerant and -sensitive cultivars of rice and wheat, respectively, following exposure to prolonged moderate heat stress regimes (Table 2). The up-regulation of HSPs at high temperatures was identified in anthers of N22 rice, a heat-tolerant cultivar, suggesting that acquired thermotolerance is important in the reproductive stage of rice development [60]. Although we classified this type of acquired thermotolerance as TMHT due to continuous exposure to moderately high temperatures it remains to be seen whether TMHT and SAT are distinguishable in crop plants grown under field conditions.

Other assays including cellular membrane thermal stability, the level of heat treatment causing continuous membrane leakage, and triphenyltetrazolium chloride reduction by heat-treated tissues as an index of cell viability [61–65] have also been used to assess thermotolerance in crop plants. Quantification of electrolyte leakage has been used to evaluate the genotypic contributions to high temperature responses in heat tolerant cultivars of both wheat and cotton [64,66,67]. Although the triphenyltetrazolium chloride reduction assay has been useful in several crop studies this approach is not commonly used in molecular genetic heat stress response studies. Chlorophyll accumulation has been used as an output of SAT for peanut and wheat leaves (Table 2). Finally, thermally-induced changes in photosynthetic parameters including maximum photochemical efficiency (Fv/Fm) and CO_2 assimilation have also been used to assess thermotolerance of heat stressed leaves (Table 2).

Recently, quantitative trait loci (QTL) mapping has been used to identify specific chromosome segments that contain candidate genes for heat tolerance [68–70]. The power of this approach to separate heat from other stresses is nicely illustrated in a recent wheat study which investigated the effects of drought and irrigation on yield. Over 100 QTLs influencing wheat yield under drought and heat stress were identified. Seventeen were associated with both stresses while 16 were exclusively associated with heat stress, demonstrating that this approach can be used to dissect complicated stress traits [71]. Even without determining the chromosomal locations of heat stress genes in crop plants, molecular genotyping technologies such as restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites, and single-nucleotide polymorphisms (SNPs) have been used to estimate the genetic contributions underlying variation in crop plant heat tolerance [67,70,72–74].

4. Conclusions and prospects

Four distinct types of plant thermotolerance have been identified in Arabidopsis, suggesting that plants use diverse mechanisms in response to temperature changes in environment. We propose the term 'thermotolerance diversity' to describe these multiple mechanisms. Distinguishing between these types of thermotolerance is important for understanding how plants respond to heat stress. Recent work in Arabidopsis has described the functions of genes required for one or more than one type of thermotolerance (Table 1), and we expect this list to become longer as more researchers start to assay for all types of thermotolerance on a routine basis.

The same approaches should be used when assaying the effects of transgenes, as it is important to know whether they confer a specific type or a wide spectrum of thermotolerance and whether they cause defects in other types of thermotolerance. Similarly, multiple heat stress phenotypes need to be assayed when studying crop plants. The complete understanding of heat stress response generated by multiple types of thermotolerance data may provide important insights for enhancing overall thermotolerance in crop plants. It will be of great interest to see whether, with more detailed thermotolerance data, tailor-made thermotolerance types can be bred or engineered in crop plants in anticipation of future climate changes.

Although we have only identified four major types of thermotolerance based on phenotyping studies, it is likely that each type can be further divided into different subtypes of thermotolerance over finer temperature ranges. For example, TMHT at 35–37 °C may be conferred by a different set of genes than at 30–32 °C. Multiple mechanisms may exist for each kind of thermotolerance, and this may allow plants to fine tune their responses to high temperatures. Similar possibilities exist for other types of thermotolerance and a detailed description of thermotolerance diversity awaits elegantly designed phenotyping experiments. The effects of combination of heat stress and other stresses such as drought, high light levels, and UV are not discussed in this review. However, it is likely that heat stress response genes are involved in both thermotolerance as well as other stress responses [3,9,75–77]. Since plants are often challenged by multiple stresses in natural environments it will be necessary to perform thermotolerance phenotyping with multiple stresses to identify and understand these interactions. Likewise, multiple thermotolerance assays should be conducted to examine whether transgenes that enhance tolerance to environmental stresses such as drought and salt result in decreased thermotolerance.

Although Arabidopsis has many advantages for these kinds of studies, recent work in the unicellular algae *Chlamydomonas reinhardtii* [78] and the moss *Physcomitrella patens* [79] have demonstrated the power of these genetic systems for understanding evolutionarily conserved components in plant heat stress response. Because these organisms are more amenable for functional genomic studies [80–82] they should be instrumental in defining new paradigms in thermotolerance diversity.

Why and how stress proteins participate in different types of thermotolerance is only clear in a small number of cases. It is likely that the mode of action of a stress protein is essential for a subset of heat stress conditions. For example, HSP101, a molecular machine involved in protein disaggregation [39,40,83] is required for tolerance to severe heat stresses but not for chronic heat stress at moderately high temperatures [15,39]. Severe heat stress induces protein unfolding and aggregation while moderate temperatures

probably do not. The use of *in vivo* assays for mis-folded proteins such as monitoring luciferase activity [40] allows ideas like this to be directly verified.

Some stress genes and their paralogs might have developed specialized roles for specific types of thermotolerance after genome duplication and subsequent subfunctionalization [9,11,15,84]. Recently we have begun to understand the differential roles of *HSF* family genes in thermotolerance diversity. In Arabidopsis the master regulators of heat stress responses, the transcription factors HSFA1A/B/D in Arabidopsis, play major roles in four different types of thermotolerance [9]. HSFA1A in tomato plays the same dominant role in BT, SAT, and TMHT [55]. In contrast, HSFA2 amplifies and prolongs the heat stress response, thus affecting LAT more than SAT [4,11,34,85]. The functions of HSFA3 and HSFA7A have been reported for BT and SAT [86,87]. Their roles in the other two types of thermotolerance remain to be examined. Intriguingly, HSFB1 and HSFB2B, two transcriptional repressors [88], were recently shown to exert opposite effects on BT and acquired thermotolerance. Double KO mutants of these genes have higher BT levels but lower levels of SAT and LAT than that of the wild type [89]. HSFA2, HSFA3, HSFA7A, HSFB1, and HSFB2B are down stream genes of HSFA1A/B/D [9,10], indicating a complex regulatory network of heat stress response that confer differential protection to a wide range of heat stress regimes. Given the large number of genes implicated in heat stress responses and the complexity of these responses there is still much to be learned before we have a good understanding of how plant respond to high temperatures. Thermotolerance diversity is a new concept that should assist us in achieving this goal.

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