

Exploring the Temperature-Stress Metabolome of *Arabidopsis*^{1[w]}

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Metabolic profiling analyses were performed to determine metabolite temporal dynamics associated with the induction of acquired thermotolerance in response to heat shock and acquired freezing tolerance in response to cold shock. Low- M_r polar metabolite analyses were performed using gas chromatography-mass spectrometry. Eighty-one identified metabolites and 416 unidentified mass spectral tags, characterized by retention time indices and specific mass fragments, were monitored. Cold shock influenced metabolism far more profoundly than heat shock. The steady-state pool sizes of 143 and 311 metabolites or mass spectral tags were altered in response to heat and cold shock, respectively. Comparison of heat- and cold-shock response patterns revealed that the majority of heat-shock responses were shared with cold-shock responses, a previously unknown relationship. Coordinate increases in the pool sizes of amino acids derived from pyruvate and oxaloacetate, polyamine precursors, and compatible solutes were observed during both heat and cold shock. In addition, many of the metabolites that showed increases in response to both heat and cold shock in this study were previously unlinked with temperature stress. This investigation provides new insight into the mechanisms of plant adaptation to thermal stress at the metabolite level, reveals relationships between heat- and cold-shock responses, and highlights the roles of known signaling molecules and protectants.

Environmental stresses arise from conditions that are unfavorable for the optimal growth and development of organisms (Levitt, 1972; Guy, 1999). Environmental stresses can be classified either as abiotic or biotic. Abiotic stresses are produced by inappropriate levels of physical components of the environment, including temperature extremes. Biotic stresses are caused by pathogens, parasites, predators, and other competing organisms. Even though biotic and abiotic stresses cause injury through unique mechanisms that result in specific responses, all forms of stress seem to elicit a common set of responses (Levitt, 1972). For instance, both biotic and abiotic stresses can result in oxidative stress through the formation of free radicals, which are highly destructive to lipids, nucleic acids, and proteins (Mittler, 2002). Another example is water stress, which is produced as a secondary stress by

chilling, freezing, heat, and salt, as a tertiary stress by radiation, and, of course, as a primary stress during drought (Levitt, 1972).

The ability of most organisms to survive and recover from unfavorable conditions is a function of basal and acquired tolerance mechanisms. Acquired tolerance involves a set of mechanisms that can transiently extend or improve overall stress tolerance (Levitt, 1972; Hallberg et al., 1985; Guy, 1999; Thomashow, 1999) following exposure to moderate stress conditions. For example, if plants are preexposed to a nonlethal high temperature, they can acquire enhanced tolerance to otherwise lethal high temperatures. Similarly, many plants can tolerate a greater level of freezing stress when they are preexposed to nonlethal low temperatures. The ability to acquire enhanced tolerance to heat stress is known as acquired thermotolerance, while enhanced tolerance to freezing could be termed acquired freezing tolerance (Guy et al., 1985; Hallberg et al., 1985; Guy, 1999; Thomashow, 1999).

It has long been suspected and is now well accepted that temperature acclimation results from a complex process involving a number of physiological and biochemical changes, including changes in membrane structure and function, tissue water content, global gene expression, protein, lipid, and primary and secondary metabolite composition (Levitt, 1972; Gilmour et al., 2000; Shinozaki and Dennis, 2003). Recent advances in genome sequencing and global gene expression analysis techniques have further established the

¹ This work was supported by National Aeronautics and Space Administration (grant no. NAG10-316), by the U.S. Department of Agriculture (National Research Initiative grant nos. 2000-35100-9532 and 2002-35100-12110), and by the Institute of Food and Agricultural Sciences at the University of Florida. This article is Journal Series Number R-10483.

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[w] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.052142.

multigenic quality of environmental stress responses and the complex nature of temperature acclimation (Seki et al., 2001; Fowler and Thomashow, 2002; Kreps et al., 2002). Literally hundreds of genes have been linked with environmental stress responses. By contrast, less is known about stress responses of plants at the metabolite and metabolome level (Cook et al., 2004; Rizhsky et al., 2004).

Global metabolite profiling analysis holds the promise to permit simultaneous monitoring of precursors, intermediates, and products of metabolic pathways. It is a discovery tool that can detect and monitor unidentified mass spectral tags (MSTs) as well as identified metabolites that play important roles in metabolism and physiology and, in the context of this work, stress tolerance. We have performed metabolite profiling analysis using gas chromatography-mass spectrometry (GC-MS) to determine similarities and differences in temporal metabolite responses and to identify novel compounds that exhibit temperature-specific responses during the induction of acquired thermotolerance in response to heat shock (HS), and during induction of acquired freezing tolerance in response to cold shock (CS). Metabolite profiling has revealed that CS influenced metabolism more profoundly than HS. However, the majority of HS responses were shared with CS, uncovering a novel relationship between HS and CS responses not previously known. This investigation provides a new viewpoint regarding metabolomic mechanisms of plant adaptation to thermal stress.

RESULTS

Temperature-Stress Acclimation Trends

Basal heat-stress tolerance for *Arabidopsis thaliana* aerial tissues was between 43°C and 44°C, using an immersion assay that was chosen to minimize experimental variation due to the influence of transpirational leaf cooling (Gates, 1968). Upon transfer to an environment with an ambient air temperature of 40°C, *Arabidopsis* shoots began to undergo induction of acquired thermotolerance. Within 15 min of exposure to 40°C, shoot thermotolerance had increased by 1°C (Fig. 1A), and over a 4-h period tissue thermotolerance increased by as much as 5°C.

Basal freezing tolerance for *Arabidopsis* was -4°C when grown in a controlled environment at 20°C. Upon exposure to 4°C, freezing tolerance increased from -4° to -11°C over the course of 96 h (Fig. 1B). Enhanced freezing tolerance was observed as early as 6 h and continued to increase until 96 h of exposure. Freezing tolerance gradually diminished after 96 h. By contrast, plants returned to 20°C after 96 h of exposure to 4°C (Fig. 1B) underwent a process known as deacclimation (DA), leading to a significant decline in freezing tolerance. Approximately one-half of the induced freezing tolerance was lost within 24 h of return to 20°C.

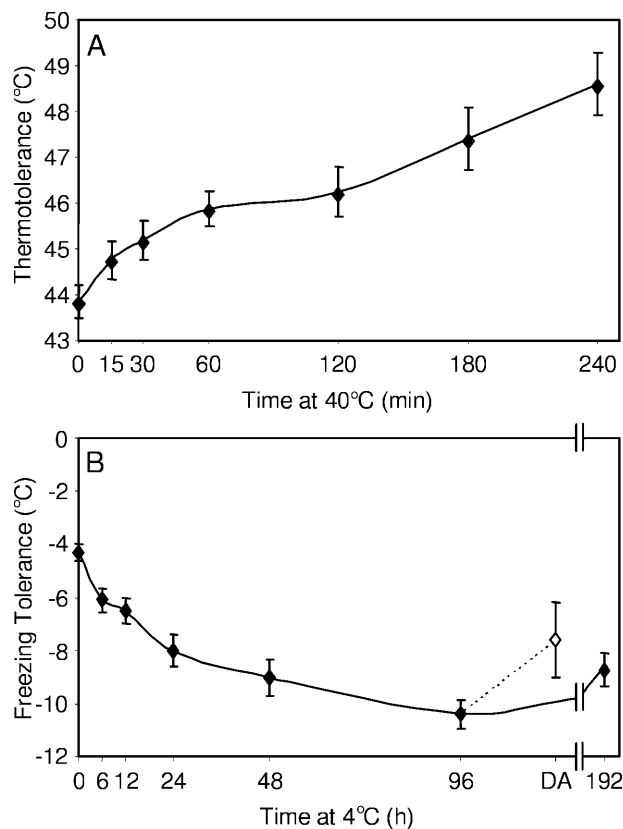


Figure 1. Kinetics of acquired thermotolerance and freezing tolerance induction in *Arabidopsis*. A, HS; B, CS. White symbol in B represents 24-h DA at 20°C after 96 h of CS. Error bars represent the 95% confidence interval of the mean. Electrolyte leakage assays were used to determine temperature causing lethal injury for acquired thermotolerance and acquired freezing tolerance.

Principal Component Analysis

Principal component analysis (PCA) was performed to test for the presence of differences between HS and CS, assess overall experimental variation, and determine individual time-point variation. PCA revealed that the four highest ranking components accounted for 61% of the total variance within the dataset (Fig. 2; Supplemental Table I, available at www.plantphysiol.org). Inspection of three of these components allowed consistent classification of the different treatment/time-point samples:

- (1) Differential response to HS and CS. The first principal component (Fig. 2), accounting for 42.1% of the variance, indicated a strong differential response of HS and CS at the metabolic level.
- (2) Differential response with respect to the time series. The second component (Fig. 2), accounting for 8.5% of total variance, resolved the time series of both HS and CS responses. The heat response followed a continuous linear trend, whereas samples of the cold-response time series were arranged in a bipartite but continuous sequence, indicative of continuous transient changes during cold

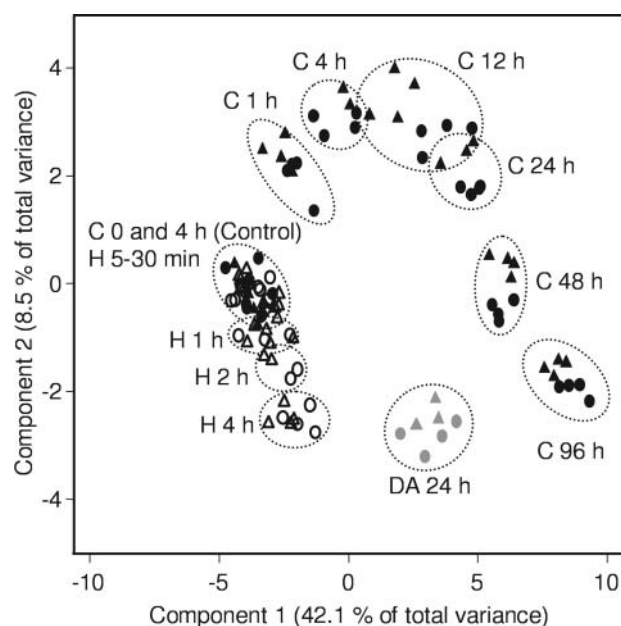


Figure 2. Principal component analysis. Component 1 (differential response to HS and CS) and Component 2 (differential response with respect to the time series) are plotted on the axes. All samples of this investigation are represented. CS samples (black), HS samples (white), untreated control samples (black), DA samples (gray), first experiment (triangles), and second experiment (circles) are shown. Time points are indicated within the graph.

acclimation. Early cold responses were opposite to the heat response, whereas late cold and heat responses were colinear.

- (3) Differential response of DA. The third component, accounting for 5.9% of total variance (Supplemental Table I), separated deacclimated samples from all other samples and clearly established their distinct metabolic phenotype compared to controls.
- (4) Component 4 and all subsequent components did not provide any further differentiation between sample types.

PCA analysis showed that the temperature treatment and time series effects clearly contributed most to the total variance within the data set. By contrast, the within-time point variance was low, as only slight shifts within the time sequence were observed (Fig. 2). The negligible interexperimental variation demonstrates the robustness of the experimental design.

Temporal Alterations of Metabolite Content in Response to Temperature Shock

We investigated sustained and transient changes with respect to three major categories of temporal response: early, intermediate, and late. Statistical analysis was performed on known metabolites and MSTs. Metabolites and MSTs were screened for significant changes ($P < 0.05$) in at least one time point after either heat or cold treatment. Of the 497 low- M_r polar

compounds detected, the levels of 143 were altered in response to HS (Supplemental Table II), and the levels of 311 were changed in response to CS (Supplemental Table III).

Out of the 143 HS-responsive metabolites and MSTs, 85 showed a sustained (Fig. 3, A, C, E, and F) or transient (Fig. 3, B and D) increase or decrease (Supplemental Table II). The majority of the metabolite responses to high temperature occurred within the first 30 min, when thermotolerance was increasing (Fig. 1A). A total of 58 metabolites and MSTs showed an early, 9 showed an intermediate, and 18 showed a late increase or decrease in response to HS. Components of amino acid and carbohydrate metabolism were affected by HS. Coordinate increases in the pool sizes of a number of amino acids (Asn, Leu, Ile, Thr, Ala, Leu, and Val) derived from oxaloacetate and pyruvate were observed (Fig. 3A). Not surprisingly, fumarate and malate (oxaloacetate precursors) contents were similarly increased. Also, a small group of

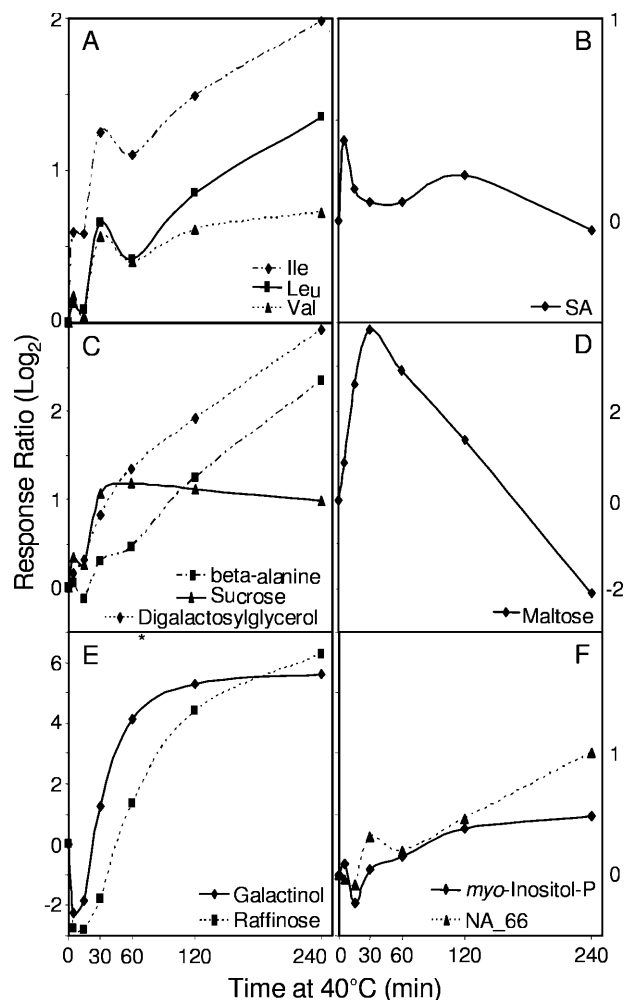


Figure 3. Representative HS metabolite responses. A to D demonstrate early; E, intermediate; F, late sustained and transient increase patterns. NA followed by number is an unidentified MST. If a tentative identification is available, MST is characterized with an asterisk.

amine-containing metabolites (β -Ala, 4-aminobutyric acid [GABA], and putrescine) with protective properties appeared to be coordinately increased. Further, a select group of well-known carbohydrates were affected (Fig. 3, C, D, E, and F), such as maltose, Suc, raffinose, its precursors galactinol, myoinositol, and cell-wall monosaccharides.

In contrast with HS, alterations in metabolite and MST contents were evenly distributed across all temporal stages of CS (Fig. 1B). Out of 311 CS-responsive compounds, 229 showed a clear sustained (Fig. 4, A, C,

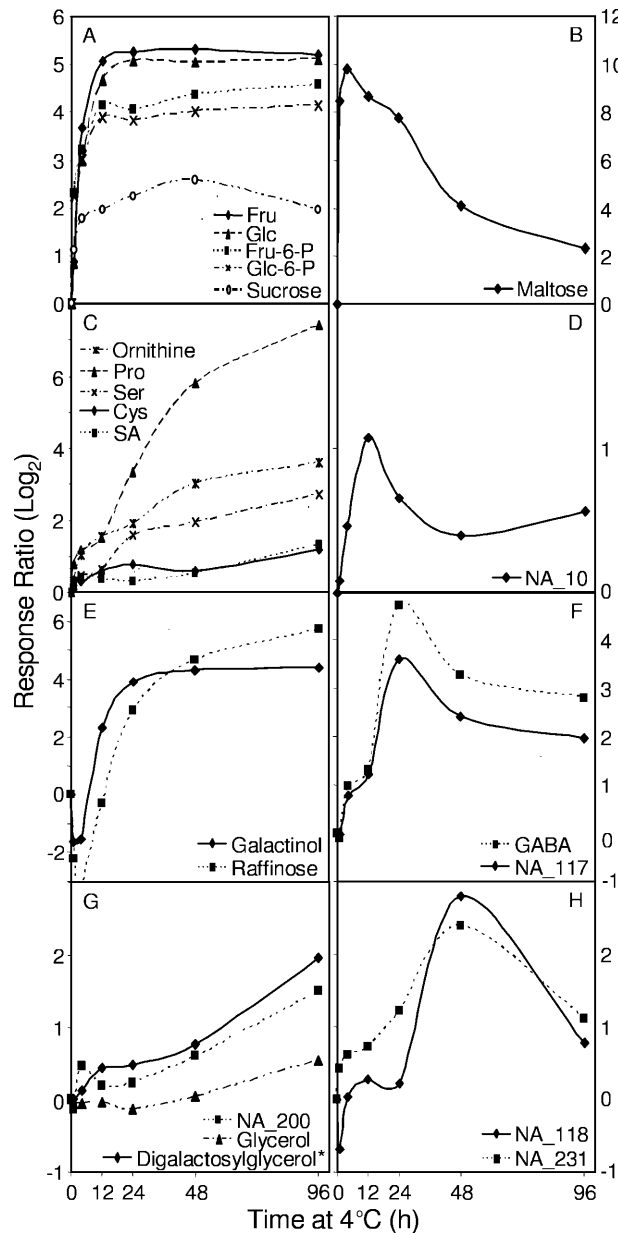


Figure 4. Representative CS metabolite responses. A to C demonstrate early; D to F, intermediate; G and H, late sustained and transient increase patterns. NA followed by number is an unidentified MST. If a tentative identification is available, MST is characterized with an asterisk.

E, and G) or transient (Fig. 4, B, D, F, and H) increase or decrease (Supplemental Table III). The pool sizes of 92 metabolites and MSTs showed an early, 66 showed an intermediate, and 71 showed a late increase or decrease to CS. Overall, amino acids, TCA cycle intermediates, and many metabolites of carbohydrate metabolism were affected by CS. Parallel to HS, coordinate increases in the pool sizes of amino acids derived from oxaloacetate and pyruvate were observed during CS. Coordinate increases in the pool sizes of aromatic amino acids (Trp, Phe, and Tyr) were followed by increased pool sizes of phenylpropanoid pathway intermediates (cis-ferulic, cis-sinapic, and trans-sinapic acid). In addition, the pool sizes of amino acids (Pro, Arg, Cys, Gly, and Ser) derived from α -ketoglutarate and from 3-phosphoglycerate were also increased. Particularly during CS, the pool sizes of most TCA cycle intermediates, as were early glycolytic intermediates, were increased. Regarding the latter, there was a clear and profound shift in hexose metabolism that linked with di- and trisaccharide accumulations (Glc, Fru, Glc-6-P, Fru-6-P, myoinositol-P, Man-6-P, galactinol, Suc, and raffinose).

Specific Temperature-Shock Responses

In order to determine similarities and differences between HS and CS responses, individual metabolites and MST profiles were compared and contrasted. Metabolites and MSTs exhibiting heat-specific (4%), cold-specific (38%), DA-specific (2%), and both HS and CS responses (25%) were identified (Fig. 5). About 31% of the metabolites and MSTs did not respond to either form of temperature shock.

Metabolites and MSTs that showed altered levels during HS, but not to CS, were considered HS specific. Eighteen compounds appeared to be heat specific (Table I), and three were identified (uracil, citramalate, and quinic acid).

Metabolites and MSTs that showed altered concentrations to CS but did not show significant changes during HS were considered CS specific. Of the 311 metabolites that responded to CS, the majority (186) was not responsive to HS (Table I). Of the 186 CS-specific metabolites, the levels of 140 increased, while 46 decreased. CS-specific metabolites included aromatic amino acids (Phe and Trp), intermediates in the phenylpropanoid pathway, α -ketoglutarate, 3-phosphoglycerate derivative amino acids, and some of the early intermediates of the glycolytic pathway.

The levels of 12 MSTs increased in response to DA but were not altered in response to HS or CS, suggesting their direct involvement in the recovery process from long-term cold stress.

A total of 125 metabolite levels were altered in response to both HS and CS, 32 exhibited a differential and 93 exhibited a common response. Of the 32, the levels of 7 metabolites decreased during HS (Table I) but increased during CS. The levels of the remaining 25 metabolites (Table I) increased when exposed to HS but

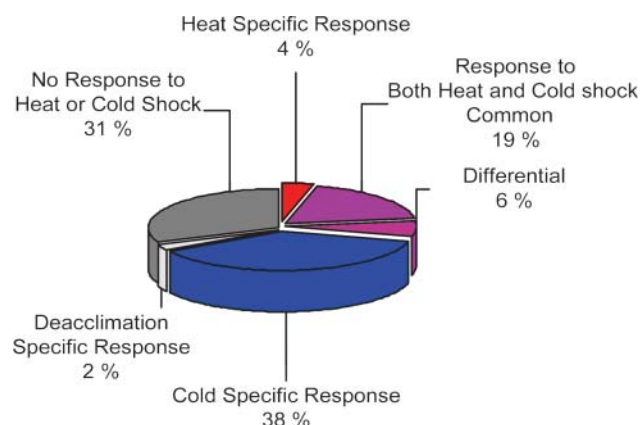


Figure 5. Proportionality of metabolite responses to temperature shock. A total of 497 metabolites and MSTs were detected by GC-MS.

decreased during CS. The 93 metabolites and MSTs that exhibited a common response to HS and CS represented two-thirds of the heat-responsive and one-third of the cold-responsive metabolites. The levels of 42 metabolites and 44 MSTs increased, while 7 decreased (Table I). Contents of oxaloacetate and pyruvate derivative amino acids, polyamines, and carbohydrates were increased under both HS and CS. A number of the metabolites in this group either have compatible solute properties or serve as precursors for secondary metabolites that protect plants against pathogens.

Common Stress Metabolites

In order to determine whether common temperature-response metabolites may play a role in overall environmental stress tolerance, the 42 metabolites with known identity were compared to those in the published literature for abiotic and biotic stress (Table I). As expected, many of them were reported to increase in response to other environmental stresses (shown with asterisk in Table I). Examples include salicylic acid (SA), GABA, Tyr, Leu, Val, Suc, and maltose (Srivastava et al., 1980; Handa et al., 1983; Mayer et al., 1990; Metraux et al., 1990; Malamy et al., 1990; Fan et al., 1993; Schmelz et al., 2003; Rizhsky et al., 2004).

The levels of the remaining 14 metabolites (Table I) were not previously reported to increase in response to temperature stress or any other environmental stresses. This grouping of 14 represents a metabolite discovery approach in the context of establishing a linkage in temperature-stress responses. The veracity of the linkage of these 14 metabolites with temperature stress was validated by a recent study (Cook et al., 2004) linking 9 of the metabolites with long-term cold acclimation of *Arabidopsis*, while this study was in review.

Statistical Linkage of Key Metabolites in the Metabolite Profile with Acquired Tolerances

In order to further investigate the contributors of the components of PCA, the metabolite loadings in com-

ponents 1, 2, 3, and 4 were compared with the ANOVA results. Based on ANOVA, the 19 metabolites and MSTs that showed quantitative signal increases in component 1 mainly consisted of metabolites that increased in response to CS. The signature metabolites for component 1 were Glu, Pro, Arg, Fru-6-P, and MSTs ([NA_154], [949; glucopyranose], [861; glucopyranose], and [539; Phe]). Component 2 was mainly composed of metabolites that showed common or differential response to both HS and CS, or cold-specific metabolites. The signature metabolites for the common response were maltose, galactinol, and raffinose; for the differential response glc, glc-6-P, and MSTs ([NA_1] and [852; aminomalonic acid]); and for the cold-specific response Pro, Fru-6-P, and MSTs ([612; Pro], [NA_84], and [NA_154]). Component 3 was mainly composed of metabolites that quantitatively increased in response to CS and decreased to control levels when deacclimated for 24 h. The signature metabolites here were galactinol, raffinose, and MSTs ([NA_154] and [497; gluconic acid 1,4 lactone]). Component 4 largely extracted metabolites that did not change quantitatively in response to either HS or CS, and they were all MSTs ([NA_159], [NA_264], [NA_267], [NA_271], [NA_302], [NA_335], [NA_341], [NA_359], and [674; Gln]). Taken together, these metabolites and MSTs of components 1 to 3 are likely to play either a direct role in essential mechanisms of acquired tolerances or an indirect role as a consequence of occupying a central role in some aspects of cell metabolism.

DISCUSSION

Metabolites have a number of functions in addition to those of intermediary metabolism. They act as signaling/regulatory agents, compatible solutes, antioxidants, or in defense against pathogens. Our results provide new insight into mechanisms of plant adaptation to thermal stress at the metabolite level, highlight the roles of known signaling molecules and protectants, and reveal a previously unrecognized interrelationship of HS and CS responses.

Plants have several well-known regulatory metabolites that function in a number of plant growth and development processes. Some are also involved in plant environmental stress processes (Klee, 2003). In this study, SA levels showed a very rapid transient increase in response to HS at 5 min (Fig. 3B). Similarly, SA levels became elevated during CS, exhibiting a biphasic response starting at 1 h, peaking at 4 and 12 h, decreasing at 24 h, and then continuously increasing to 96 h (Fig. 4C). These findings firmly implicate SA as an early signaling molecule in temperature-stress responses. This is important for a number of reasons. SA has a key role in systemic acquired resistance to pathogens such as bacteria, fungi, and viruses (Metraux et al., 1990; Schmelz et al., 2003), and increases in SA levels are positively correlated with the

Table 1. Influence of temperature shock on metabolite levels

I, Increase; D, decrease; N, no significant change in metabolite concentration. Names in brackets precede a match value for an unidentified compound. These names indicate best mass spectral similarity on a scale of 0 to 1,000 (1,000 is identical) to the indicated compound. *, Metabolite showed increase in other environmental stress conditions.

| HS-Specific Response | | | Differential Response | | |
|----------------------------------|----|----|-----------------------------|----|----|
| Metabolites | HS | CS | Metabolites | HS | CS |
| Uracil | I | N | Phosphoric acid | D | I |
| D-(–)-Quinic acid | I | N | Glc | D | I |
| 13 MSTs | I | N | Glc-6-P | D | I |
| Citramalic acid | D | N | [798; Fru] | D | I |
| 3 MSTs | D | N | 3 MSTs | D | I |
| CS-Specific Response | | | Common Response | | |
| Allantoin | N | I | 2-Ketoglutaric acid | I | I |
| <i>cis</i> -Aconitic acid | N | I | β -Ala* | I | I |
| <i>cis</i> -Ferulic acid | N | I | Citric acid* | I | I |
| <i>cis</i> -Sinapic acid | N | I | Erythritol | I | I |
| Fru-6-P | N | I | Erythronic acid | I | I |
| GlcUA | N | I | Fru* | I | I |
| Glyceric acid-3-P | N | I | Fumaric acid | I | I |
| L-(+)-Ascorbic acid* | N | I | GABA* | I | I |
| L-Arg* | N | I | Galactinol* | I | I |
| L-Cys* | N | I | Galactonic acid | I | I |
| L-Glu* | N | I | Glycerol* | I | I |
| L-Gln* | N | I | Gly* | I | I |
| L-Phe* | N | I | L-Ala* | I | I |
| L-Pro* | N | I | L-Asn* | I | I |
| L-Ser* | N | I | L-Glycerol-3-P | I | I |
| L-Trp* | N | I | L-HomoSer* | I | I |
| Maleic acid | N | I | L-Ile* | I | I |
| Man-6-P | N | I | L-Leu* | I | I |
| Norvaline | N | I | L-Lys* | I | I |
| O-Acetyl-L-Ser | N | I | L-Met* | I | I |
| Octadecanoic acid | N | I | L-Thr* | I | I |
| PyroGlu | N | I | L-Tyr* | I | I |
| Sorbitol* | N | I | L-Valine* | I | I |
| <i>trans</i> -Sinapic acid | N | I | Malic acid* | I | I |
| [497; Gluconic acid-1,4-lactone] | N | I | Maltose* | I | I |
| [529; Indole-3-acetic acid] | N | I | Melibiose* | I | I |
| [539; Phe] | N | I | Myoinositol-P | I | I |
| [612; Pro] | N | I | Orn | I | I |
| [614; Gln] | N | I | Putrescine* | I | I |
| [640; Putrescine] | N | I | Raffinose* | I | I |
| [734; L-Asp] | N | I | Ribose | I | I |
| [861; Glucopyranose] | N | I | SA* | I | I |
| [889; 1,6-AnhydroGlc] | N | I | Succinic acid | I | I |
| [949; Glucopyranose] | N | I | Suc* | I | I |
| 106 MSTs | N | I | Threonic acid | I | I |
| Isocitric acid | N | D | Threonic acid-1,4-lactone | I | I |
| Lactic acid | N | D | Trehalose* | I | I |
| 44 MSTs | N | D | Tyramine | I | I |
| Differential Response | | | Xyl | I | I |
| Ara | I | D | [721; Glucaric acid] | I | I |
| Man | I | D | [732; Pipecolic acid] | I | I |
| <i>myo</i> -inositol | I | D | [861; Digalactosylglycerol] | I | I |
| Shikimic acid | I | D | 44 MSTs | I | I |
| [852; Aminomalonic acid] | I | D | Dehydroascorbic acid dimer | D | D |
| [708; Ribonic acid] | I | D | Glyceric acid | D | D |
| [846; Xylitol] | I | D | L-Asp | D | D |
| 18 MSTs | I | D | 4 MSTs | D | D |

level of resistance to pathogens in plants (Heil and Bostock, 2002). Accordingly, a recent study has linked SA with basal thermotolerance (Clarke et al., 2004), and exogenous application of SA or acetyl salicylate has been shown to enhance thermotolerance (Dat et al., 1998; Lopez-Delgado et al., 1998; Senaratna et al., 2000; Clarke et al., 2004). One study has shown endogenous SA levels to be elevated at 30 min after the onset of HS (Dat et al., 1998). Our findings place the increase in SA levels to within 5 min of the onset of HS, which strongly implies a role for SA in early HS-signaling and acquired thermotolerance. Further, increases in SA levels during temperature shock in this study and in other abiotic (drought and salt stress) and biotic stresses (Garcia et al., 1997; Metraux et al., 1990; Munne-Bosch and Penuelas, 2003; Schmelz et al., 2003) suggest that SA could be a key signal molecule in the initiation of plant tolerance to a variety of environmental stresses. It is logical that integrating SA signaling in temperature-shock responses could help plants prepare to defend themselves against pathogens when plant host-pathogen defense systems are weakened by environmental stress.

Many metabolites can act in defense mechanisms against pests such as insects, pathogenic fungi, and bacteria. These metabolites are generally derived from secondary metabolism, such as the phenylpropanoid, isoprenoid, alkaloid, or fatty acid/polyketide pathways (Dixon, 2001). However, precursors of these defense compounds emanate from primary metabolism. For example, branched-chain amino acids (Ile, Leu, and Val) serve as precursors for cyanogenic glycosides (Vetter, 2000). Aromatic amino acids (Trp, Phe, and Tyr) serve as precursors for indole glucosinolates, phytoalexins, alkaloids, lignins, flavonoids, isoflavonoids, and hydroxycinnamic acids (Dixon, 2001). In this study, increased levels of Ile, Leu, Val, and Tyr in response to both HS and CS (Table I; Fig. 3A) and increased levels of Trp and Phe in response to CS were observed (Table I). The increase in Ile, Leu, Val, and Tyr content in response to other abiotic and biotic stresses and heat is well known (Srivastava et al., 1980; Mayer et al., 1990; Rizhsky et al., 2004). Therefore, it is reasonable that one purpose for branched-chain amino acid accumulation is to support increased production of secondary metabolites as part of a defense response against pathogens during temperature stress. Such a response could constitute a preemptive defense against opportunistic attack by a pathogen on a stress-weakened host.

Metabolites of primary metabolism can act as signal molecules. A well-known example is Suc (Koch, 1996; Chiou and Bush, 1998; Roitsch, 1999; Smeekens, 2000; Rolland et al., 2002; Moore et al., 2003), whose content in response to HS (within 5 min) and CS rose very rapidly (within 1 h) and was maintained throughout HS and CS exposures (Figs. 3B and 4A). Thus, Suc could be a candidate signaling molecule for both HS and CS, based on its early accumulation in response to temperature shock. Consistent with this notion, par-

allel microarray analysis has revealed that the promoters of a number of genes induced by both HS and CS contain sugar-responsive elements (F. Kaplan, D.Y. Sung, and C.L. Guy, unpublished data). Thus, a reasonable hypothesis that sugar signaling may be important in the establishment and maintenance of both acquired thermotolerance and freezing tolerance is worthy of further study. Arabidopsis knockouts defective in sugar signaling might prove valuable experimental tools in dissection of the signaling aspects of sugars during acquired thermotolerance and freezing tolerance. In addition to signaling role of Suc, the role of Suc and other soluble sugars (maltose, Glc, and Fru) as compatible solutes are well established during abiotic stresses, such as cold, drought, desiccation, salt, and osmotic stress (Guy et al., 1992; Fan et al., 1993; Uemura et al., 2003; Rizhsky et al., 2004).

These findings support the notion that a multiplicity of primary metabolites could act collectively as compatible solutes. Compatible solutes (osmolytes, osmoprotectants) are low- M_r organic molecules that accumulate under stress conditions, and are considered to stabilize proteins and membranes and contribute to cell osmotic pressure. There are three general types of osmoprotectants: amino acids, quaternary ammonium compounds, and polyols (Bowlus and Somero, 1979; Yancey et al., 1982; Shahjee et al., 2002). During the onset of acquired thermotolerance, the content of Ala, Asn, β -Ala, Fru, GABA, glycerol, malate, maltose, Man, putrescine, raffinose, succinate, Suc, and trehalose increased in response to HS. A complementary GC-MS study consistent with our study also showed that 6 h of HS resulted in moderate increases in the content of β -Ala, glycerol, maltose, Suc, and trehalose (Rizhsky et al., 2004). The content of these metabolites increased with the persistence of the HS, and their proportions to each other changed as the duration of high-temperature exposure progressed (Fig. 3). Similar to the HS response, we observed increases in the content of many metabolites with known compatible solute properties during the development of acquired freezing tolerance at low temperature (Fig. 4). Examples include Ala, β -ala, Gly, Pro, Ser, Orn, putrescine, Fru, Glc, malate, maltose, and Suc. Persistence of low-temperature exposure led to increased quantities of these compatible solute metabolites and produced the accumulation of even more metabolites with compatible solute-like properties, notably Asn, GABA, glycerol, raffinose, sorbitol, succinate, and trehalose. This overall profile of metabolites with compatible solute-like properties suggests that it is a combination of compatible solutes that exerts additive or synergistic effects during the cold acclimation process and during the induction of thermotolerance. For instance, the Arabidopsis mutant (*eskimo1*), containing high levels of soluble sugars and Pro, possesses enhanced freezing tolerance (Xin and Browse, 1998). By contrast, failure to accumulate Suc and Glc results in reduced freezing tolerance in the Arabidopsis mutant (*sfr4*; McKown et al., 1996). The

enhanced freeze sensitivity of *sfr4* was shown to be due to the loss of osmotic responsiveness (LOR) of the protoplast. When exogenous Suc was supplied in vitro, LOR was reduced and freeze tolerance was improved in *sfr4* protoplasts (Uemura et al., 2003). The present metabolite profiling results implicate a more dynamic and larger compatible solute-like network than previously recognized. This may explain why attempts to engineer overproduction of a single compatible solute compound have not produced plants with high levels of stress tolerance (Chen and Murata, 2002).

The major advantage of metabolite profiling using a time-course design is that it permits simultaneous monitoring of entire metabolic pathways (precursors, intermediates, and products) and can reveal the subtle interplay of functionally related metabolites. In this study, a clear example of substrate and product relationship can be seen in Figures 3E and 4E. Galactinol, along with Suc, is an immediate precursor of raffinose, whose biosynthesis rate largely depends on the availability of Suc, galactinol, and the enzyme raffinose synthase (Taji et al., 2002). An increase in galactinol abundance clearly precedes the increase in raffinose content during both HS and CS, exactly as would be predicted by a classic substrate-product relationship. Such parallel relationships can be very powerful as a metabolite discovery tool that detects and monitors MSTs that may play important roles in stress adaptations.

In conclusion, the comparative metabolomic analysis of temperature-stress response has highlighted the roles of signaling molecules and implicated the action of a compatible solute network in temperature-stress tolerances. With respect to low- M_r polar compounds, CS, in a quantitative sense, influenced metabolism more profoundly than HS. The majority of metabolites responsive to CS were specific to CS. By contrast, a very large proportion of the HS metabolite response (about two-thirds) seemed to be shared with that of CS. Only a very small proportion of heat-responsive metabolites were heat specific. The present results support a number of paradoxical early observations that some cold-hardened plants were also more heat-stress tolerant (Alexandrov, 1964; Levitt, 1972). These results may also explain why HS seems to improve chilling tolerance (Lurie and Klein, 1991; McCollum et al., 1995; Saltveit, 2002; Saltveit and Hepler, 2004; Saltveit et al., 2004) in a number of cold-sensitive species. Therefore, treatment by HS for short periods of time might improve tolerance to acute HS and CS or induce an overall environmental stress tolerance. Additionally, heat- and cold-shocked plants increased concentrations of a number of metabolites that responded to a variety of environmental stimuli. The majority of the common temperature shock-responsive metabolites was accumulated during all stages of the development of acquired tolerances and did not seem to be specific to one particular phase in the development of acquired tolerance. Taken together, this work identifies a large

number of potential metabolic targets for further in-depth investigations of acquired tolerances to temperature stress.

MATERIAL AND METHODS

Plant Growth

Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia) plants were grown as described by Sung and Guy (2003) for 3 weeks. Plants were grown at 20°C with a photoperiod of 15/9-h light/dark cycle in growth cabinets for 3 weeks. Irradiance was provided by incandescent bulbs and cool-white fluorescent tubes and ranged between 100 and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy height. Plants were at an eight-leaf stage of development when the experiments were begun (Supplemental Fig. 1).

Electrolyte Leakage Assay for Thermotolerance

To measure acquired thermotolerance, plants were given a HS (40°C) for 0, 15, 30, 60, 120, 180, and 240 min beginning 2 h after the onset of the light period. At the indicated times, plants were immersed to the soil lines in 42°C, 44°C, 46°C, 48°C, and 50°C water for 10 min, and electrolyte leakage of the aerial portion of a plant was measured 3 d after heat treatment (Sung and Guy, 2003). For each time point and temperature, 14 independent experiments with 3 replications were done, except for 180 and 240 min. For time points 180 and 240 min, one experiment with 5 replications was done.

For measuring freezing stress, plants were given a CS (4°C) for 0, 6, 12, 24, 48, 96, and 192 h beginning 2 h after the onset of the light period. Also, plants were deacclimated for 24 h at 20°C after 96 h of CS. At the indicated times, plants were rapidly harvested, wrapped in water-saturated tissue paper, placed in a test tube, then placed in a controlled-temperature bath (Forma Scientific model 2425; Marietta, OH) and equilibrated for 30 min at 0°C. Then a chip of ice was placed in contact with the tissue paper and the temperature was lowered at a rate of 2°C h⁻¹. Tubes were removed at 1° intervals, placed on ice, and allowed to thaw overnight at 4°C. For each time point and temperature, three independent experiments with five replications were done. For DA, one experiment with five replications was done.

Electrolyte leakage of the aerial portions of the plants was measured according to Sung and Guy (2003). Aerial portions of plants were placed in scintillation vials containing 10 mL of distilled water and shaken for 1 h. After the first conductivity reading was made, the tissue was boiled for 2 min by microwave irradiation. After cooling to room temperature, a second conductivity reading was taken following shaking for a second hour. Relative electrolyte leakage was determined from the ratio of first and second conductivity measurements.

Metabolite Profiling

Time points for metabolite profiling during temperature-shock treatments were selected based on thermo- and freeze-tolerance time-course experiments. Temperature-shock treatments were initiated 2 h after the onset of the light period, which allowed for the harvest of all samples within the light period. Three-week-old 20°C-grown plants were placed at 40°C and sampled at 5, 15, 30, 60, 120, and 240 min of HS. At the same time, a second set of plants was placed at 4°C and sampled at 1, 4, 12, 24, 48, and 96 h of CS. After 96 h at 4°C, plants were returned to 20°C and sampled after 24 h. Additionally, untreated controls were taken at zero time of the experiment and 4 h after the experiment began. All samples were rapidly harvested, flash-frozen in liquid nitrogen (<30 s), and stored at -80°C until metabolite extraction. Two sets of temperature-stress experiments were performed, each comprising two to four replicate measurements per time point. Aerial tissues were ground in liquid nitrogen with pestle and mortar. Aliquots of 60 mg of frozen powder were extracted with hot MeOH/CHCl₃ and the fraction of polar metabolites processed as described (Wagner et al., 2003). Ribitol, isoascorbic acid, and deuterated Ala were added as internal standards. A C₁₂, C₁₅, C₁₉, C₂₂, C₂₈, C₃₂, and C₃₆ *n*-alkane mixture was used for the determination of retention time indices (RI). Metabolite samples were derivatized as described by Fiehn et al. (2000) and Roessner et al. (2000), analyzed by an MD 800 GC-MS system

(ThermoQuest, Manchester, UK). Chromatograms were processed using the find algorithm of the MassLab version 1.4 software (ThermoQuest).

Metabolite Identification

GC-MS-based metabolite profiling detects and quantifies specific mass spectral fragments in defined retention time windows. Identification of these fragments was performed through standard addition experiments using pure authenticated compounds to confirm identity by retention time index and mass spectrum. Compounds were designated as metabolites if they were identified with a match >750 on a scale of 0 to 1,000 and RI deviation <3.0. Other unidentified compounds are designated as MSTs designated by the code NA and a unique number. In cases of high mass spectral similarity of MSTs to available commercial or custom mass spectral libraries, MSTs were named in square brackets by a preceding match value and a compound name taken from these libraries. Representative mass spectra and RI, which serve for metabolite identification in Arabidopsis, and novel identifications post-publication will be available through CSBDB (<http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html>).

Statistical Analysis

PCA was performed with the S-Plus 2000 software package standard edition release 3 (Insightful, Berlin) on \log_{10} -transformed relative responses, $\log_{10}(R_i)$. Missing data were replaced with 0 for PCA. The denominator of the quotient, R_i , was the average response of nontreated control samples at zero time of the respective stress experiments ($R_i = N_i \times \text{avg}N_{i0}^{-1}$). Responses (N_i) were volume corrected for error during sample preparation or GC injection and normalized by the fresh weight of each sample.

One-way ANOVA was done using the Kruskal-Wallis test on metabolite response values (N_i). Nonparametric approach was chosen because it did not require normally distributed data, and it was also more resistant to the outliers in the data set that might lead to high fold changes. Changes in metabolite content with $P < 0.05$ were considered to be significant. Pair-wise comparisons between different treatments and time points were done using the Kruskal-Wallis test.

Classification Criteria of Metabolite Responses

Criteria for HS metabolic responses were as follows: in the early sustained response (0–4 h), a statistically significant change in metabolite levels as compared to zero-time control occurred at 5, 15, or 30 min and was maintained until 4 h. In the intermediate-sustained response (1–4 h), a statistically significant response occurred at either 1 or 2 h, was maintained until 4 h, but did not exhibit a significant response at 5, 15, and 30 min. In the late response (4 h), a statistically significant response occurred at 4 h, but no significant response was observed at 5, 15, 30, 60, and 120 min. In the transient response, compounds exhibited a statistically significant response when compared to zero-time control, 4-h diurnal control, and 4-h HS. Transient changes occurring at 5, 15, and 30 min were considered early transient, and those at 1 and 2 h were considered intermediate transient. Additionally, a 4-h untreated control was included in the analysis to filter diurnal responses from the HS data set.

The criteria for the CS metabolic responses were adjusted based on the Arrhenius equation relationship for respiratory processes (Yelenosky and Guy, 1977) to reflect the slowed metabolism during CS as follows: in the early sustained response (0–96 h), a statistically significant change in metabolite levels as compared to zero time occurred at 1 h or 4 h and was maintained until 96 h. In the intermediate-sustained response (12–96 h), a significant response occurred at 12 or 24 h, was maintained until 96 h, but no significant response occurred at 1 and 4 h. In the late response (48–96 h), a significant response occurred at either 48 or 96 h, but no significant response was observed at 1, 4, 12, and 24 h. In the transient response, metabolites showed a statistically significant response when compared to zero-time control and 96-h CS. Transient changes occurring at 1 and 4 h were considered early transient, those at 12 and 24 h were considered intermediate transient, and those at 48 h were considered late transient. Diurnally regulated metabolites were filtered from the 4-h early transient response group using the 4-h diurnal (untreated) control. Metabolites that did not fit the above criteria were classified as increase or decrease if they showed a significant increase or decrease at any time point during temperature shock when compared to zero-time control.

ACKNOWLEDGMENTS

We thank Michael Popp and Kil-Jae Lee for their help with this work, and B. Rathinasabathi, K.C. Cline, M.F. Thomashow, and Lonnie Ingram for critical reading of the manuscript. We also thank Lothar Willmitzer and Max Planck Society for continuing support.

Received August 23, 2004; returned for revision October 5, 2004; accepted October 5, 2004.

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