Additive Effects of Alcohols and Polyols on Thermostability of Pepper Leaf Extracts

Jeffrey A. Anderson

Department of Horticulture and Landscape Architecture, Oklahoma State Univ., 360 Agriculture Hall, Stillwater, OK 74078-6027

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ABSTRACT. Chemical chaperones (CC) are plant stress-related compounds that can stabilize protein structure in adverse environments. Modes of action are thought to involve hydrogen bonding, primarily with the solvent, and hydrophobic stabilization of the protein core. The objective of this study was to determine structure–function relationships between CC (and structurally related compounds) and thermal stability of pepper (*Capsicum annuum* L.) leaf proteins. Both polarity [based on log K_{ow} (the oil–water partition coefficient)] and capacity for hydrogen bonding (based on the number of OH groups) contributed to whether low-molecular-weight alcohols and polyols stabilized or destabilized proteins at elevated temperatures. Thermal stability increased with increasing number of OH groups at a fixed number of carbon atoms per molecule. Conversely, thermal stability decreased with increasing number of carbon atoms with a fixed number of OH groups. When CC solution concentrations were adjusted to the same concentration of OH groups (1.51×10^{22} OH groups per milliliter), protein thermal stability increased with increasing CC polarity. Mixtures of different CC had additive effects on increasing protein thermostability, but mixtures of stabilizing (mannitol) and destabilizing (methanol) compounds negated each other. As a strategy for increasing plant thermotolerance, identification and removal of destabilizing compounds should be equally effective as increasing levels of stabilizers in protecting protein conformation at elevated temperatures.

Two primary focal points in abiotic stress tolerance are heat shock proteins and compatible solutes. Compatible solutes have been implicated in resistance to water deficit, salt, and temperature stresses and can accumulate to high concentrations without disrupting protein structure. Chemical chaperones (CC), including sugars, polyols, amino acids, and methylamines, are a subset of compatible solutes capable of stabilizing protein structure. Some of the more extensively studied compounds with CC activity include mannitol (Ruijter et al., 2003), glycerol (Kim and Lee, 1993), trehalose (Kaushik and Bhat, 2003), maltose (Kaplan and Guy, 2004), sucrose (Millard et al., 2003), glycine betaine (Chow et al., 2001), and trimethylamine oxide (Bennion and Daggett, 2004). A number of organisms accumulate CC to stabilize protein structure during stresses that promote denaturation (Managbanag and Torzilli, 2002). Trehalose plays a significant role in acquisition of heat tolerance in *Saccharomyces cerevisiae* Meyen ex. E.C. Hansen (Hottiger et al., 1989). *Aspergillus niger* van Tieghem conidiospores from a mutant deficient in mannitol production were heat-sensitive (Ruijter et al., 2003). A protective role was confirmed when exogenous mannitol restored thermotolerance in the mutants.

Evidence for protein stabilization by CC has been amply demonstrated, but the mode of action is subject to several interpretations (Ganea and Harding, 2005). This is not surprising because protein unfolding by chemical denaturants is also an incompletely understood phenomenon (Bennion and

Daggett, 2004). Although heat shock proteins and CC can protect proteins from denaturation, the mechanisms differ. Some families of heat shock proteins bind to exposed hydrophobic patches, preventing aggregation or misfolding of partially unfolded proteins (“molecular chaperone” activity) (Jacob et al., 1995). Chemical chaperones appear to maintain native protein structure through interactions with the protein and solvent but may have some benefit in reducing aggregation of unfolded proteins. Although the precise nature of the interaction among protein, CC, and solvent is not clear, it is likely to be affected by chemical properties of each (Kaushik and Bhat, 2003). One way to address this dilemma is to use a complex mixture of proteins that represents a range in protein physical properties to assess CC action (Owusu and Cowan, 1989). Although information on CC activity in complex mixtures is lacking, a number of researchers have explored stabilization of individual proteins. Within limits, protein stability increased with increasing concentration (Back et al., 1979) and size (Davis-Searles et al., 2001) of polyol.

Although significant progress has been made in modeling protein–solvent–CC interactions, this remains a controversial area. One approach is to view CC as a subset of cosolvents that exhibit a continuum of colligative and noncolligative effects on protein structure and function (Davis-Searles et al., 2001). Cosolvents can be considered destabilizing or stabilizing depending, in part, on the relative affinity of protein for the cosolvent and water. Attempts to model and quantify CC action include both interactions at the protein surface (preferential solvation) and changes in free energy associated with exposing the hydrophobic core on unfolding. If the driving forces for protein–solvent–cosolvent interactions are a composite of the changes in free energy associated with replacing individual solvent molecules with cosolvent molecules, mixtures of different cosolvents should have additive effects on protein stability.

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Correspondence. E-mail: jeff.anderson@okstate.edu

The objective of the present study was to use a protein thermostability assay developed using pepper leaf extracts (Anderson, 2006) to evaluate the protein stabilizing/destabilizing effects of series of structurally related compounds. Properties of compounds examined are listed in Table 1. Structure–function relationships of CC were explored by correlating thermoprotective capacity with indices of polarity and OH group density. Mixtures of cosolvents were used to test the hypothesis that cosolvents have additive effects on protein stabilization.

Materials and Methods

PLANT CULTURE. ‘Early Calwonder’ pepper seeds were planted in 24-cm-diameter pots in an enriched commercial potting mix (BM-1; Saint-Modeste, Quebec). Amendments included dolomite (3.6 g·L⁻¹), triple superphosphate (0.7 g·L⁻¹), Micromax (The Scotts Co., Marysville, Ohio) (0.6 g·L⁻¹), and KNO₃ (0.6 g·L⁻¹). Plants were grown in a controlled-environment chamber (model PGW36; Conviron, Winnipeg, Man., Canada) at 24/20 °C day–night cycles with 45% to 65% relative humidity. The chamber was programmed for 14-h photoperiods with a photosynthetic photon flux (PPF) density at canopy height of ≈400 μmol·m⁻²·s⁻¹. Plants were watered with soluble fertilizer (20N–8.6P–16.6K, Peters; The Scotts Co.) at 0.7 g·L⁻¹ as required based on lighter growing substrate color and reduced pot weight relative to plants not requiring irrigation.

PLANT EXTRACTS AND HEAT TREATMENTS. Fully expanded, nonsenescent leaves were excised from 10-week-old plants and rinsed in deionized water and then gently blotted free of surface moisture. The midribs were removed with a razor blade and the leaves were cut into sections. Leaf extracts were prepared at 62.5 mg FW per milliliter of 2-(N-morpholino) ethanesulfonic acid [MES (50 mM, pH 6.0)] buffer containing 1 mM ethylenediaminetetraacetic acid with 1.25 g polyvinylpolypyrrolidone in both the blender and filtration collection flask as previously described (Anderson, 2006). After centrifugation at 16,000 g_n for 20 min at 21 °C, the supernatant was diluted 1:1 (v/v) with buffer (control) or 2× solutions of test compounds yielding 31.25 mg FW leaf tissue per milliliter of solution. Supernatants were transferred to test tubes (3.4 mL in each tube) with three tubes (subsamples) per exposure temperature by treatment combination. Tubes were held in circulating baths

at the appropriate test temperatures for 15 min and then placed in a water bath at 21 °C. The turbidity-based protein thermostability assay measured apparent absorbance at 540 nm using a 4-h period between temperature treatments and spectrophotometric measurements. Nonreplicated preliminary experiments based on visual observations instead of spectrophotometric measurements were conducted to determine treatment temperature ranges that included apparent absorbance maxima for each cosolvent. An exception was n-propyl alcohol, which caused increased absorbance at 21 °C when used at 500 mM. All reagents were obtained from Sigma-Aldrich Co. (St. Louis), except for ethylenediaminetetraacetic acid and MES, which were purchased from Fisher Scientific (Fair Lawn, N.J.).

FIXED OH GROUP TO CARBON ATOM RATIO WITH INCREASING MOLECULAR WEIGHT. Leaf extract was diluted 1:1 (v/v) with buffer (control), or 1-M solutions of methanol, ethylene glycol, glycerol, or mannitol prepared in buffer, yielding 0.5-M solutions. Solutions were exposed for 15 min to 21 °C (control) or 44 to 54 °C in 1 °C increments.

RANGE IN NUMBER OF OH GROUPS WITH FIXED NUMBER OF CARBON ATOMS. Leaf extract was diluted 1:1 (v/v) with buffer (control), 1-M solutions of n-propyl alcohol, propylene glycol, or glycerol prepared in buffer yielding 0.5-M solutions. Solutions were exposed for 15 min to 21 °C (control) or 44 to 53 °C in 1 °C increments.

RANGE IN NUMBER OF CARBON ATOMS WITH FIXED NUMBER OF OH GROUPS. Leaf extract was diluted 1:1 (v/v) with buffer (control), or 0.2-M solutions of methanol, ethanol, n-propyl alcohol, or n-butyl alcohol prepared in buffer yielding 0.1-M solutions. Solutions were exposed for 15 min to 21 °C (control) or 43 to 52 °C in 1 °C increments.

FIXED OH GROUP DENSITY WITH A RANGE IN POLARITY. Leaf extract was diluted 1:1 (v/v) with buffer (control), or solutions of methanol (2.03 M), propylene glycol (1.85 M), ethylene glycol (1.40 M), glycerol (1.22 M), or mannitol (1.00 M) prepared in buffer yielding solutions containing 1.51 × 10²² OH groups per milliliter. Concentration of OH groups was calculated by dividing the number of OH groups per mole by the molar volume and then multiplying by the solution concentration. Solutions were exposed for 15 min to 21 °C (control) or 36 to 54 °C in 2 °C increments.

MIXTURES OF STABILIZING AND DESTABILIZING COSOLVENTS. Leaf extract was diluted 1:1 (v/v) with buffer (control), or solutions of methanol or mannitol individually, or in combination. Final concentrations were 500 mM methanol, 500 mM mannitol, 500 mM methanol plus 500 mM mannitol and 250 mM methanol plus 250 mM mannitol. Solutions were exposed for 15 min to 21 °C (control) or 45 to 54 °C in 1 °C increments.

MIXTURES OF STABILIZING COSOLVENTS. Leaf extract was diluted 1:1 (v/v) with buffer (control), 2× solutions of glucose or mannitol individually, or in combination. Final concentrations were 250 mM glucose, 250 mM mannitol, 250 mM glucose plus 250 mM mannitol, and 125 mM glucose plus 125 mM mannitol. Solutions were exposed for 15 min to 21 °C (control) or 46 to 54 °C in 1 °C increments.

QUANTIFICATION OF STRUCTURE–FUNCTION RELATIONSHIPS. The relationships between hydrogen bonding potential or polarity and protein thermostability were determined by plotting the treatment temperature yielding the maximum apparent absorbance versus OH group density or versus polarity based on the oil/water partition coefficient, log Kow (Sangster, 2006), of

Table 1. Properties of compounds used in pepper leaf thermostability experiments.^z

Cosolvent	Molecular wt	Molecular formula	Log Kow	OH groups per milliliter × 10 ⁻²²
Methanol	32.04	CH ₄ O	-0.74	1.49
Ethanol	46.07	C ₂ H ₆ O	-0.30	1.03
Propanol	60.09	C ₃ H ₈ O	0.25	0.81
Ethylene glycol	62.07	C ₂ H ₆ O ₂	-1.36	2.16
Butanol	74.12	C ₄ H ₁₀ O	0.84	0.66
Propylene glycol	76.09	C ₃ H ₈ O ₂	-0.92	1.64
Glycerol	92.09	C ₃ H ₈ O ₃	-1.76	2.47
Glucose	180.16	C ₆ H ₁₂ O ₆	-3.24	3.08
Mannitol	182.17	C ₆ H ₁₄ O ₆	-3.10	3.00

^zLog Kow values are derived from oil–water partition coefficients. The number of OH groups per milliliter is reported for a 1.0-M solution.

the alcohols and polyols from three of the first four experiments described. Similar analysis was not possible with the experiment examining *n*-propyl alcohol, propylene glycol, and glycerol because a maximum apparent absorbance temperature could not be determined from this experiment for the 500 mM propanol solution. Controls, which lacked added alcohols or polyols, were not included in regression analyses of cosolvents. Least-squares regression equations were calculated for linear and second-degree polynomial relationships between the variables.

All experiments were conducted independently on three dates with three subsamples, except for the experiments with equal OH group density that were conducted on four dates with two subsamples per treatment–temperature combination. Analysis of variance was conducted using PROC GLM (SAS Institute, Cary, N.C.). A highly significant ($P \leq 0.01$) treatment $\times$ exposure temperature interaction was observed in all series of experiments. Therefore, mean separation was conducted within exposure temperature by Duncan's multiple range test at $P \leq 0.05$ using treatment $\times$ date as the error term.

Results

FIXED OH GROUP TO CARBON ATOM RATIO WITH INCREASING MOLECULAR WEIGHT. Thermal stability of extracts increased with increasing molecular weight in compounds with a consistent carbon atom to OH group ratio (Fig. 1). Mannitol stabilized the leaf extract, resulting in a maximum apparent absorbance temperature 2 °C higher than the control. Apparent absorbance of the 500 mM glycerol solution was not significantly different from the control at 50 °C. Ethylene glycol and methanol solutions exhibited maximum apparent absorbance temperatures 1 and 3 °C lower than the control, respectively.

RANGE IN NUMBER OF OH GROUPS WITH FIXED NUMBER OF CARBON ATOMS. Thermal stability of extracts increased with increasing number of OH groups in compounds with three carbon atoms (Fig. 2). Glycerol, with three OH groups, had a maximum apparent absorbance temperature ≈ 1 °C higher than the control, and propylene glycol (two OH groups) had a maximum apparent absorbance temperature ≈ 1 °C lower than the control. The maximum apparent absorbance temperature for the 500 mM *n*-propyl alcohol solution could not be

Fig. 1. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 44 to 54 °C in 1 °C increments for 15 min for pepper leaf extracts containing only buffer (control) or 500 mM of methanol, ethylene glycol, glycerol, or mannitol. Error bars represent $\pm$ SE.

Fig. 2. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 44 to 53 °C in 1 °C increments for 15 min for pepper leaf extracts containing only buffer (control) or 500 mM of *n*-propyl alcohol, propylene glycol, or glycerol. Error bars represent $\pm$ SE.

determined because the solution was sufficiently destabilized to form a precipitate by 44 °C.

RANGE IN NUMBER OF CARBON ATOMS WITH FIXED NUMBER OF OH GROUPS. The maximum apparent absorbance temperature decreased with increasing number of carbon atoms in alcohols with one to four carbons (Fig. 3). At 100 mM, the methanol solution was similar in thermostability to the control. Ethanol and propanol lowered the maximum apparent absorbance temperature 1 and 2 °C, respectively. Butanol exhibited a maximum apparent absorbance temperature ≤ 43 °C.

FIXED OH GROUP DENSITY WITH A RANGE IN POLARITY. Although precision was reduced by using 2 °C intervals, maximum apparent absorbance temperatures increased through a series of compounds with increasing polarity but equal OH group density (Fig. 4). Methanol, the least hydrophilic cosolvent in this group (Table 1), had a maximum apparent absorbance temperature of 44 °C, and mannitol, the most hydrophilic cosolvent, had a maximum apparent absorbance temperature of 52 °C. Direct comparisons of relative CC activity could not be made in this experiment because cosolvents were used at different concentrations.

MIXTURES OF STABILIZING AND DESTABILIZING COSOLVENTS. Methanol destabilized the leaf extract by 2 °C and mannitol

Fig. 3. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 43 to 52 °C in 1 °C increments for 15 min for pepper leaf extracts containing only buffer (control) or 100 mM of methanol, ethanol, propanol, or butanol. Error bars represent $\pm$ SE.

Fig. 4. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 36 to 54 °C in 2 °C increments for 15 min for pepper leaf extracts containing only buffer (control) or methanol (1015 mM), propylene glycol (925 mM), ethylene glycol (700 mM), glycerol (610 mM), or mannitol (500 mM). Error bars represent $\pm$ SE.

stabilized the extract by 3 °C at 500 mM (Fig. 5). When methanol and mannitol were mixed in the same solution, the maximum apparent absorbance temperature was not significantly different from the control. The negating effects were observed at both 250- and 500-mM solutions of both compounds.

MIXTURES OF STABILIZING COSOLVENTS. Mannitol or glucose, singly, at 250 mM or both compounds together at 125 mM each increased the thermal stability of leaf extracts by 1 °C compared with the control (Fig. 6). The maximum apparent absorbance temperature was 2 °C higher than the control when both compounds were present at 250 mM each.

STRUCTURE-FUNCTION RELATIONSHIPS. Relationships between protein thermostability and physical properties of alcohols and polyols were examined to gain insight into structural features that contribute to protein stability or instability at elevated temperatures. Correlation analysis between maximum apparent absorbance temperature and OH group density or log Kow for the cosolvent series methanol, ethylene

Fig. 5. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 45 to 54 °C in 1 °C increments for 15 min for pepper leaf extracts containing only buffer (control), 500 mM of methanol, 500 mM of mannitol, 250 mM of methanol plus 250 mM mannitol (M&M 250), or 500 mM of methanol plus 500 mM mannitol (M&M 500). Error bars represent $\pm$ SE.

Fig. 6. Apparent absorbance at 540 nm (AA_{540}) after exposure to 21 °C or 46 to 54 °C in 1 °C increments for 15 min for pepper leaf extracts containing only buffer (control), 250 mM of glucose, 250 mM of mannitol, 125 mM of glucose plus 125 mM mannitol (G&M 125), or 250 mM of glucose plus 250 mM mannitol (G&M 250). Error bars represent $\pm$ SE.

glycol, glycerol, and mannitol exhibited significant linear and quadratic relationships with R^2 values at or near 1.0 (Table 2). A series of alcohols (methanol, ethanol, propanol, and butanol) exhibited a similar relationship between the variables with R^2 values ranging from 0.75 to 0.99. Experiments with cosolvent concentrations adjusted to yield the same OH group density indicated significant linear and quadratic trends between maximum apparent absorbance temperature and log Kow.

Discussion

Interest in CC activity is fueled by prospects of increasing plant stress tolerance, improving the efficiency of enzymatically catalyzed reactions important in industrial applications and improving the quality of processed foods. Scientists from multiple disciplines have provided insights on protein stabilization by CC spanning from empirical to theoretical viewpoints. Although significant progress has been achieved in determining the interactions between protein, solvent, and cosolvent, gaps in our understanding persist. One difficulty involves the use of different proteins that vary considerably in physical properties. Therefore, it is not surprising that experiments reporting relative effectiveness of CC using different proteins have reported trehalose (Kaushik and Bhat, 2003), glucose (Back et al., 1979), glycerol (Guiavarc'h et al., 2003), and sarcosine (Chow et al., 2001) to be superior. The present study addresses this dilemma by using a complex mixture of proteins that more closely represents behavior of a plant cytoplasm than a solution of an individual protein.

A series of structurally related cosolvents spanning a range in polarity and OH group density exhibited continuous effects ranging from destabilization to stabilization of pepper leaf proteins at high temperatures. In general, protein thermostability increased with increasing cosolvent polarity and concentration of OH groups. Consistent with previous reports using individual proteins (Davis-Searles et al., 2001), the magnitude of the response was dependent on concentration and molecular weight of the cosolvent. The relative contributions of molecular

Table 2. Linear and quadratic relationships between maximum apparent absorbance temperature of pepper leaf extracts and OH group density or polarity [based on log Kow (the oil-water partition coefficient)] of chemical chaperones and related compounds.

Cosolvents	OH density	R ²	Log Kow	R ²
Methanol, ethylene glycol, glycerol, mannitol (500 mM)	$y = 6.51x + 42.06$ $y = 1.60x^2 + 2.92x + 43.95$	0.99 0.99	$y = -2.04x + 45.96$ $y = -0.62x^2 - 4.50x + 44.01$	0.96 1.00
Methanol, ethanol, propanol, butanol (100 mM)	$y = 5.17x + 40.85$ $y = -1169.10x^2 + 306.51x + 28.22$	0.75 0.95	$y = -3.06x + 46.04$ $y = -1.50x^2 - 2.89x + 46.56$	0.94 0.99
Methanol (1015 mM), propylene glycol (925 mM), ethylene glycol (700 mM), glycerol (610 mM), mannitol (500 mM)	NA ² NA		$y = -3.04x + 42.80$ $y = -0.69x^2 - 5.75x + 40.75$	0.93 0.95

²Not applicable because concentrations were adjusted to yield equal OH group densities.

weight and polarity on protein thermostability were not evaluated as a result of the lack of suitable compounds ranging in only a single variable. Cosolvent polarity and H-bonding potential were not independent in the alcohols and polyols examined because OH groups contributed to polarity. However, by adjusting cosolvent concentrations to result in equal OH group density (Fig. 4), results demonstrated that CC activity was not merely a function of OH density. Interpreting structure–function relationships will involve examination of the composite effects of complex, interacting variables.

By examining compounds ranging in polarity and OH group density, a point of neutrality with respect to protein thermostability was observed. At the point of neutrality, the cosolvent was not stabilizing or destabilizing compared with the solution without an added cosolvent. For example, based on the maximum apparent absorbance temperature of the control in the experiment including methanol, ethylene glycol, glycerol, and mannitol at 500 mM (Fig. 1), a point of neutrality was reached at an OH group density of $\approx 1.25 \times 10^{22}$ per milliliter and a log Kow of ≈ -2 . The observed point of neutrality was not interpreted as a constant and should vary based on the experimental parameters used. An analogy can be drawn with protein–solvent–cosolvent binding interactions, in which the point of neutrality represents equal binding affinities between the protein and solvent and between the protein and cosolvent (Timasheff, 2002). If protein–solvent–cosolvent binding principles can be extended to structural stabilization, the point of neutrality represented a functional equivalence between the solvent and cosolvent. Compounds with greater polarity or density of OH groups than the point of neutrality were superior to the aqueous solvent in maintaining protein structure at high temperatures in this system. Cosolvents with less polarity or a lower OH group density increased the free energy relative to the solvent, resulting in protein destabilization. Results cannot be extended beyond the alcohols and polyols used because structural features such as aromatic rings can dominate the chemical properties. Hydrolyzable tannins, which are usually associated with woody dicots, strongly destabilize protein structure although they contain multiple OH groups (Siebert et al., 1996). Similarly, addition of gallic or tannic acids promoted loss of protein solubility in pepper leaf extracts (J.A. Anderson, unpublished data). It is likely that any hydrolyzable tannins initially present in pepper leaf extracts were bound by polyvinylpyrrolidone used in the extraction process.

Mixtures of cosolvents exerted an additive effect on protein thermostability. Mixing the two stabilizing compounds, mannitol and glucose, increased protein thermostability as much as either compound singly at an equivalent concentration. Con-

versely, extracts containing both methanol (destabilizing) and mannitol (stabilizing) had a maximum apparent absorbance temperature similar to the control. These findings are consistent with a thermodynamic approach to describing cosolvent effects on protein thermostability based on the overall change in free energy from substituting solvent molecules with cosolvent molecules.

As a practical approach, scientists have explored increasing stress tolerance by increasing levels of compounds with CC activity. Dose–response experiments have shown that relatively high concentrations of CC are required for maximal activity. For example, the denaturation temperature of ovalbumin increased as sorbitol and sucrose concentration increased to 50% (w/w) (Back et al., 1979). Mannitol significantly increased protein stability at 500 mM but not at 50 mM (Anderson, 2006). The highest mannitol levels calculated in plants are probably in salt-adapted celery (*Apium graveolens* L.) at up to 300 mM (Stoop et al., 1996). Trehalose levels in heat-shocked yeast cytosol (≈ 500 mM) were more than sufficient to afford protein thermoprotection (Hottiger et al., 1994). Physiological levels of maltose, glucose, and trehalose having a measurable effect in protecting electron transport chain activities were reported in heat-shocked pea thylakoids (Kaplan and Guy, 2004). However, achieving beneficial CC levels with respect to thermotolerance may be limited by activity of hydrolyzing enzymes or development of abnormal phenotypes (Goddijn et al., 1997; Sebollela et al., 2004). Because cosolvents have an additive effect on protein thermostability, removal of destabilizing compounds should be equally effective as increasing levels of stabilizers. Identifying and reducing the levels of protein destabilizers, instead of generating high levels of stabilizers, should eliminate the requirement to reduce CC levels shortly after stress to prevent deleterious phenotypes.

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