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Physiological effects of azetidine on cellular leakage in soybean seedlings

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Abstract

In addition to heat shock (HS), a proline analog, azetidine-2-carboxylic acid (Aze), also induces heat shock protein (HSP) synthesis. Here, we describe our further characterization of the induced effects by Aze treatment in soybean seedlings. Northern blot analyses revealed transcription of the soybean HS genes *hsp70* and *hsc70* as well as *hspl7.5* activated after 6 h of Aze incubation, as reported previously. However, Aze-induced activation of HSP genes was not attenuated after transferring seedlings pretreated with Aze to shaking buffer, whereas the activation was decreased when seedlings were treated with Aze and then proline. Besides triggering an HS-like response, Aze treatment triggered a continuous increase of cellular leakage in soybean seedlings at room temperature. Nonlethal HS pretreatment protected seedlings from cellular leakage induced by Aze and allowed them to survive subsequent lethal HS treatment. As well, 10 mM proline prevented electrolyte leakage after 6 h of 10 mM Aze treatment and increased the survival of seedlings under lethal treatment. These results indicate that Aze treatment causes a permeability change in the plasma membrane by leading to cellular leakage before accumulation of HSPs. *In vitro* chaperone activity of 10 mM Aze-induced sHSP-CIs (class I small HSPs) with the model substrate citrate synthase was less efficient than that from 2 h of 40 °C treatment.

Keywords: Amino acid analog; Azetidine-2-carboxylic acid; Chaperone activity; Electrolyte leakage; Small heat shock proteins

1. Introduction

All organisms respond to heat shock (HS) stress by the predominant synthesis of heat shock proteins (HSPs) and repression of normal gene activity. The transcription of HSP genes is triggered by thermal misfolding of cellular proteins, which leads to the activation of HS factor binding to HS elements [1,2]. Other than HS, many agents such as amino acid analogs, arsenite, cadmium and abscisic acid activate *HSP* expression under normal temperatures [3–5]. The stresses that trigger HSP synthesis have the common property of either damaging proteins directly or causing cells to synthesize misfolded proteins so that accumulation of abnormal misfolded proteins can be one of the signals that activates *HSP* expression in cells [6,7]. The HSPs induced by canavanine, an arginine analog, are nonfunctional in *Drosophila* and mammalian cells [8,9].

In contrast to HS, amino acid analogs showed slower kinetics for HS gene activation, requiring 24 h to achieve a similar magnitude of accumulation as HSPs [10–12]. A proline analog, azetidine-2-carboxylic acid (Aze), exerted its effects on cellular protein through incorporation into newly synthesized proteins by replacing proline residues in animal cells, *Arabidopsis thaliana* and *Escherichia coli* [13–15]. Aze inhibited the growth in bacterial cells by altering the protein tertiary structure and acting as a false end-product inhibitor of proline biosynthesis [16,17]. Thus, Aze is a valuable reagent for characterizing cellular response to protein misfolding during stress conditions.

Aze is usually found in the Lilaceae family, but its physiological effect in Lilaceae plants remains unclear [18]. Aze-induced abnormal-misfolded proteins are generally thought to be the signal causing the onset of *HSP* expression in cells. However, at 28 °C, Aze cannot activate all members of small HSP (sHSP) genes in soybean seedlings [10]. In rice, four of the nine class I sHSP (sHSP-CI) genes on chromosome 3 were shown to be selectively induced by Aze [19]. So, regulatory mechanisms other than HS control the induction of sHSP genes with Aze. Additionally, little is known about the function of plant sHSPs induced by Aze in relation to acquisition of thermotolerance.

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Recently, we showed that sHSP-CIs accumulated to a level similar to that with 2 h HS in soybean (*Glycine max*) seedlings during 24 h of 10 mM Aze treatment [12]. However, with Aze-induced accumulation of sHSPs followed by lethal treatment, soybean seedlings did not acquire thermotolerance. However, seedlings pretreated with 10 mM Aze for 6 h, then transferred to a solution containing 10 mM proline for up to 18 h acquired the thermotolerance. Substituting proline with other amino acids (e.g. Gly, Phe, Cys and Gln) following 6 h Aze incubation did not induce thermotolerance [12]. In this study, we aimed to analyze Aze-induced effects on HS response in soybean. Seedlings were viable with nonlethal HS treatment prior to Aze incubation then lethal HS treatment. As well, we showed change in cellular membrane/permeability by Aze treatment as a primary effect, although induced sHSP-CIs still show chaperone activity *in vitro*.

2. Methods

2.1. Plant materials and growth analysis

Seeds of soybean (*G. max* cv Kaohsiung No.8) were allowed to germinate in rolls of moist paper towel at 28 °C in a dark growth chamber [20]. Two-day-old etiolated seedlings were incubated in shaking buffer (1 mM potassium phosphate, 1% sucrose, 50 μg/ml chloramphenicol, pH 6.0). HS treatments were as described previously [21]. Seedlings underwent nonlethal HS (40 °C for 2 h) and lethal HS (45 °C for 2 h). Seedlings for characterizing the effect of Aze on thermotolerance were incubated in shaking buffer containing 10 mM Aze and 10 mM proline as indicated. After Aze treatment, seedlings were washed thoroughly with water and then supplanted in a roll of moist paper towel at 28 °C in a dark growth chamber. Seedling length and weight were measured to determine growth after the indicated treatments.

2.2. Viability assay

Two-day-old soybean seedling samples treated with high temperature or Aze as indicated were incubated overnight in 5 ml buffer containing 50 mM potassium phosphate (pH 7.4), 0.6% 2,3,5-triphenyltetrazolium chloride (TTC) and 0.05% Tween-20 under a slight vacuum in the dark as described [22]. The seedlings were then rinsed thoroughly with water and weighed. The samples were homogenized and extracted with 95% ethanol at 100 °C for 10 min. The absorbance of each sample was monitored at 530 nm.

2.3. Electrophoresis, immunoblotting and quantitative estimation of proteins

One-dimensional SDS–PAGE was performed with 15% (w/v) polyacrylamide gels according to the method of Laemmli [23]. Proteins were transferred from polyacrylamide gels to PVDF membranes (Immobilon, Millipore) according to the manufacturer's instructions. Protein bands cross-reacting with the sHSP-CI antibodies [24] were identified by reaction with peroxidase conjugated to goat anti-rabbit IgG (Bio-Rad). Bound antibodies were visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium according to the manufacturer's specifications (Bio-Rad). The amount of protein that reacted with antibodies was determined by scanning the membranes with a densitometer (model SI, Molecular Dunamics, Sunnyvale, CA) and use of ImageQuant software (Molecular Dynamics). The proteins were quantified by Bradford assay [25].

2.4. Northern hybridization analysis

Total RNAs isolated from soybean seedlings were separated by electrophoresis in 1% agarose gels containing 10 mM morpholinopropanesulfonate and 6% formaldehyde (pH 8.0) and blotted onto nitrocellulose papers [20]. The cDNA clones used for making probes were pCE53, hsp70 and hsc70 encoding HSP17.5, HSP70 and HSC70, respectively [26–28]. Probes for Northern blots were prepared by random primer labeling of cDNA inserts with [α-^{32}P]dCTP (GE Healthcare Bio-Sciences) as described [20]. All blots were prehybridized for 4 h in hybridization buffer (50% formamide, 6× SSC, 0.5% SDS, 0.1% Ficoll, 0.1% PVP, 0.1% BSA, and 100 μg/ml denatured sheared-salmon sperm DNA). A ^{32}P-labeled probe was added into the hybridization buffer, and blots were hybridized overnight at 42 °C and washed as described [20].

2.5. Purification of the sHSP complex

The sHSP-CI complex induced by nonlethal HS and Aze (10 mM for 24 h) was purified according to the method of Yeh et al. [29]. After nonlethal HS and Aze treatment, seedlings were harvested and homogenized in buffer containing 200 mM Tris (pH 7.8), 100 mM KC1, 30 mM $MgCl_2$, 500 mM sucrose, 1 mM DTT and 1 mM PMSF. The homogenate underwent centrifugation for 90 min at 300,000 × *g* at 4 °C for preparation of the postribosomal supernatant (PRS). The PRS was fractionated by 70–100% saturation with ammonium sulfate. The precipitate was pelleted, dissolved in buffer containing 50 mM Tris (pH 8.8), 1 mM EDTA and 0.1% β-mercaptoethanol, and dialyzed overnight against the same buffer at 4 °C. The protein fraction was subjected to a nondenaturing linear gradient gel (5–20% acrylamide gel). The protein band equivalent to the sHSP-CI complex was cut off and eluted by use of an Electro-Eluter (Bio-Rad, model L422).

2.6. Chaperone activity analysis

Thermal aggregation suppression analysis was performed as described previously [30] with porcine heart citrate synthase (CS; Sigma) used as a substrate. An amount of 75 nM of CS (monomer concentration) was incubated in the presence or absence of 50–100 nM of the isolated HSP complex (monomer concentration) from HS- or Aze-treated soybean seedlings in 50 mM potassium buffer, pH 7.5 (800 μl total). Samples were monitored for light scattering at 320 nm in a Hitachi U3200 spectrophotometer with a thermostated cell compartment preheated at 43 °C.

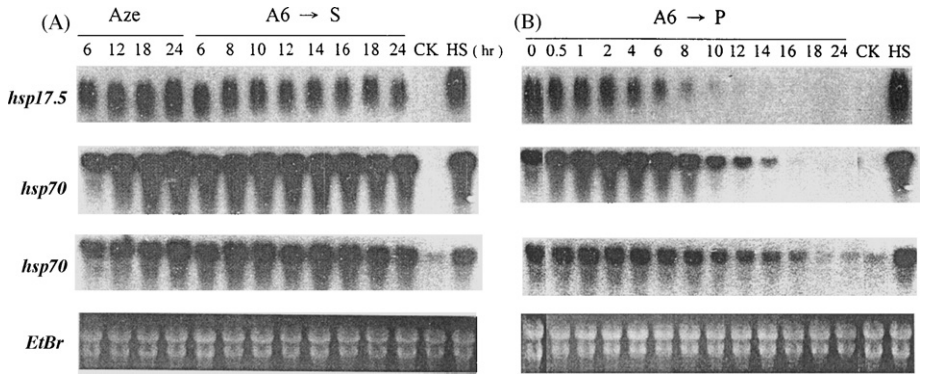

Fig. 1. (A and B) Characterization of expression of Aze-inducible HSP genes. A total of 15 μg of total RNA from soybean seedlings treated as indicated was applied to each lane. The ^{32}P-labeled cDNA probes used are indicated to the left of each panel. A6, 6 h of 10 mM Aze treatment; S, shaking buffer; P, 10 mM proline; CK, 28 °C; HS, 40 °C for 2 h. An ethidium bromide-stained gel (EtBr) was used to show equal loading.

2.7. Measurement of conductivity

Ten etiolated soybean seedlings with cotyledons removed were incubated in deionized water with or without the addition of Aze at 28 or 40 °C. Leakage of electrolytes into the incubation medium was determined by use of a conductivity meter (Jenway 4010, Jenway Ltd., Dunmow, Essex, UK) as described [21].

3. Results

3.1. Expression and function of Aze-induced HSPs

We examined the effect of Aze treatment on accumulation of HS mRNA and chaperone activity of sHSP-CIs. The mRNA level of *hspl7.5*, *hsp70*, and *hsc70* accumulated significantly after 6 h 10 mM Aze treatment (Fig. 1A). The transcript level of HS genes was sustained (*hspl7.5* and *hsc70*) or slightly increased (*hsp70*) during 24 h Aze treatment (Fig. 1A). Interestingly, after transferring 6 h Aze-pretreated seedlings to shaking buffer with or without proline, HS mRNA was still detectable after recovery of 6 h Aze treatment but decreased greatly with Aze followed by proline treatment (Fig. 1B). The level of *hspl7.5* mRNA was undetectable after 10 h of proline replacement. These results suggest that Aze induced an irreversible signal to activate HS genes, and proline could antagonize the Aze effect.

Soybean sHSPs function as molecular chaperones [31]. The multimerization of sHSPs is a prerequisite for the chaperone/thermoprotection function [31,32]. Immunoblot analysis showed that a high-molecular-mass complex formed by Aze-induced sHSP-CI expression at 28 °C was similar in size to that formed by 2 h of continuous 40 °C treatment [12,33]. Our previous data showed that this protein complex could stabilize total soluble proteins *in vitro* but was not sufficient to confer thermotolerance *in vivo* [12]. Here, we further compared the chaperone activity of the complexes from 24 h Aze-treated seedlings and nonlethal HS-treated seedlings using the model substrate citrate synthase. Light scattering by citrate synthase aggregation could be detected at 43 °C but was suppressed in the presence of 50 or 100 nM of HSP complex (60 or 80% reduction, respectively) from nonlethal HS-treated seedlings (Fig. 2). However, 50 nM of sHSP-CI complex was not able to act efficiently on suppression of CS thermal aggregation (35% reduction), although 100 nM of sHSP-CI complex from seedlings treated with Aze alone or a mixture of protein complexes from nonlethal HS- and Aze-treated seedlings could prevent 80% of citrate synthase aggregation under thermal stress. We previously reported that Aze was not incorporated into de novo synthesized sHSP-CIs [12]. The sHSP-CI complex provide substantial thermostabilization of soluble proteins against heat denaturation at 55 °C for 30 min, and the degree of protection is proportional to the amount of complex added [12]. This finding clearly indicates that Aze treatment did not result in dysfunction of sHSPs *in vitro*.

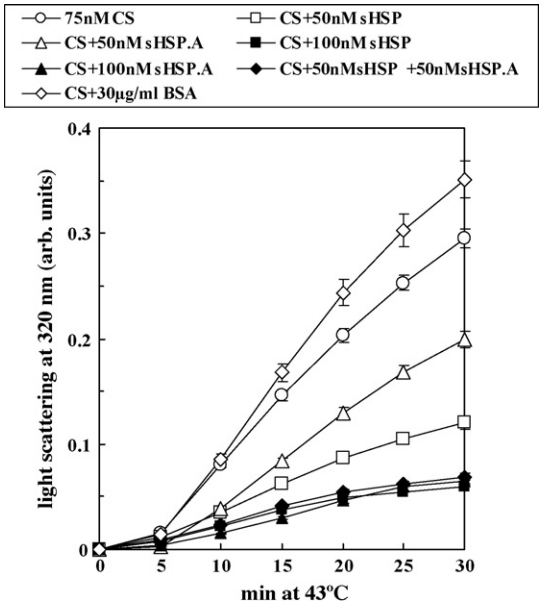

Fig. 2. *In vitro* chaperone activity of Aze-induced sHSPs. Citrate synthase (CS; 75 nM) in the absence or presence of purified sHSP complexes induced by nonlethal HS (sHSPs; 40 °C for 2 h) and Aze alone (sHSPs.A; 10 mM Aze for 24 h) as described was incubated at 43 °C for up to 30 min. Samples were taken for light scattering at 320 nm. CS alone (○), CS + 50 nM sHSPs (□), CS + 100 nM sHSPs (■), CS + 50 nM sHSPs.A (△), CS + 100 nM sHSPs.A (▲) and CS + 50 nM sHSPs + 50 nM sHSPs.A (◆). CS incubated with 30 μg/ml BSA (◇) was also tested for light scattering as a control.

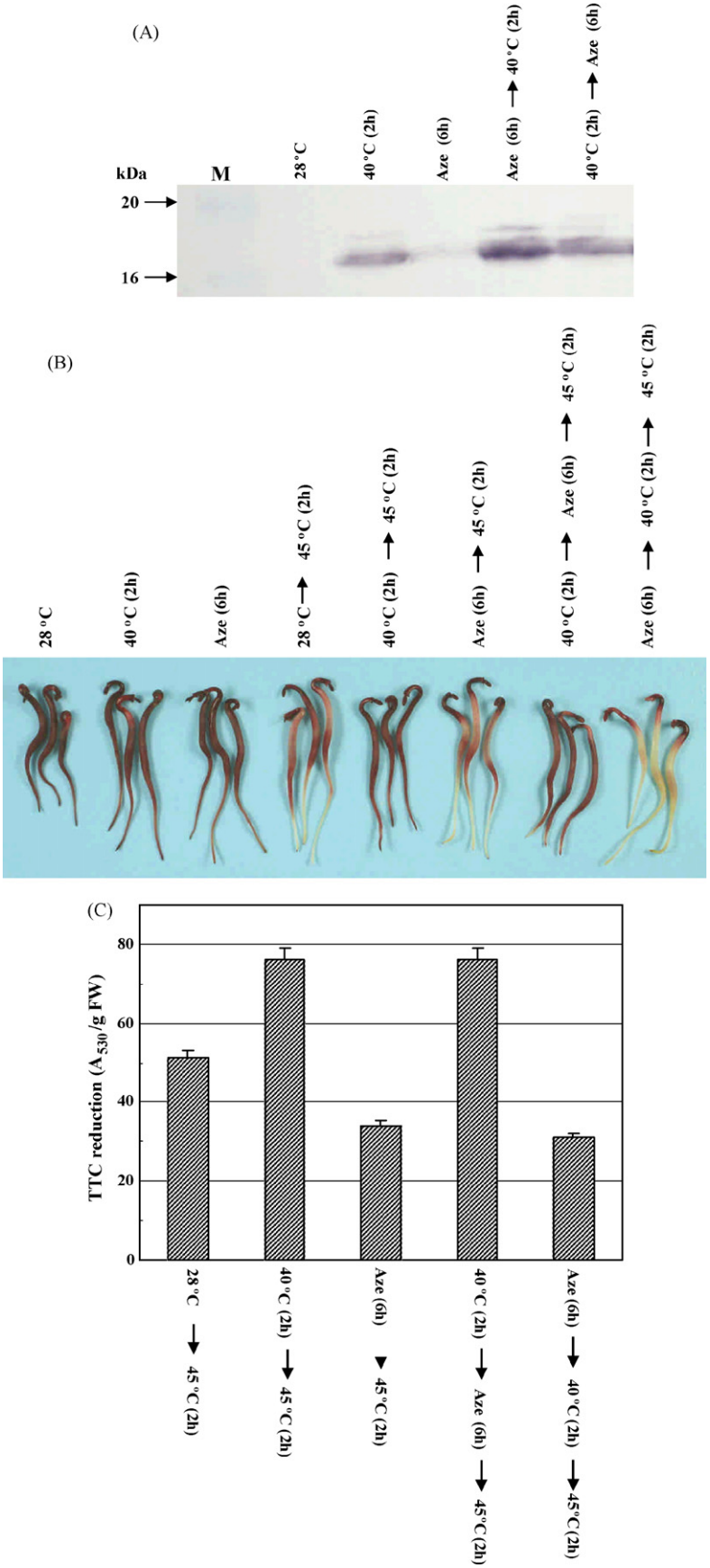

Fig. 3. Viability of seedlings treated with nonlethal HS (40 °C for 2 h) and 10 mM Aze (28 °C). (A) Immunoblot analysis of soybean sHSP-CIs. Total protein was extracted from seedlings after treatments as indicated. A total of 20 μg of protein was loaded in each lane and subsequently immunoblotting using sHSP-CI

3.2. Thermotolerance of Aze-treated soybean seedlings with prior nonlethal HS

We showed previously that Aze-induced sHSP-CIs were neither accumulated in the soluble fraction nor associated with conferring thermotolerance in soybean seedlings, although the sHSP-CIs induced by Aze were functional in protecting soluble proteins from heat denaturation [12]. Regarding the effect of Aze on thermotolerance to lethal treatment, we compared soybean seedlings treated with nonlethal HS prior to Aze treatment, to Aze treatment followed by nonlethal HS, under lethal treatment. Seedling viability was determined by TTC staining. Viable cells following TTC treatment are able to reduce colorless TTC compound to deep red Formazan [34]. In contrast, dead cells are not stained. TTC staining revealed the viability of soybean seedlings and was consistent with the viability confirmed by seedling growth [20]. Seedlings were viable with nonlethal HS treatment for 2 h prior to 6 h Aze incubation then lethal HS treatment, whereas seedlings with 6 h Aze treatment or 6 h Aze treatment followed by 2 h of 40 °C were sensitive to lethal HS stress (Fig. 3B). To confirm the result shown in Fig. 3B, we used seedlings to quantify TTC reduction activity after the indicated treatment. After seedlings were transferred to lethal treatment, the value of absorbance at 530 nm (A_{530}/g FW) of seedlings treated with nonlethal HS followed by Aze was approximately two-fold higher than that of the seedlings treated with 6 h Aze or 6 h Aze followed by 2 h of 40 °C (Fig. 3C). Western blot analysis detected sHSP-CIs after 6 h of 10 mM Aze treatment (Fig. 3A). As well, accumulation of sHSP-CIs in response to nonlethal HS treatment prior to or after 6 h Aze incubation was slightly higher than that with nonlethal HS treatment alone. We also examined the effect of other stress agents (e.g. arsenite and cadmium) prior to 6 h Aze treatment before lethal HS stress. The above pretreatments could not improve thermotolerance of seedlings to lethal HS stress as judged by TTC staining (data not shown). These results suggest that the pre-accumulation of HSPs induced by HS pretreatment could confer resistance to the detrimental effects of Aze and to the lethal heat stress.

3.3. Aze treatment causing continuous membrane leakage can be rescued by nonlethal HS treatment

Aze has been shown to be transported into cells via proline transporters, which causes the misfolding of proteins and eventually inhibits cellular growth in *A. thaliana* and yeast cells [35,36]. The shift in localization of Aze-induced sHSP-CIs was found in soybean seedlings [12]. Therefore, the cellular physiological condition of soybean seedlings was altered under Aze treatment. Here we found that HS pretreatment can overcome the Aze effects in soybean seedlings. To further characterize the Aze effects, we next investigated the effects of Aze treatment on electrolyte leakage in soybean seedlings at

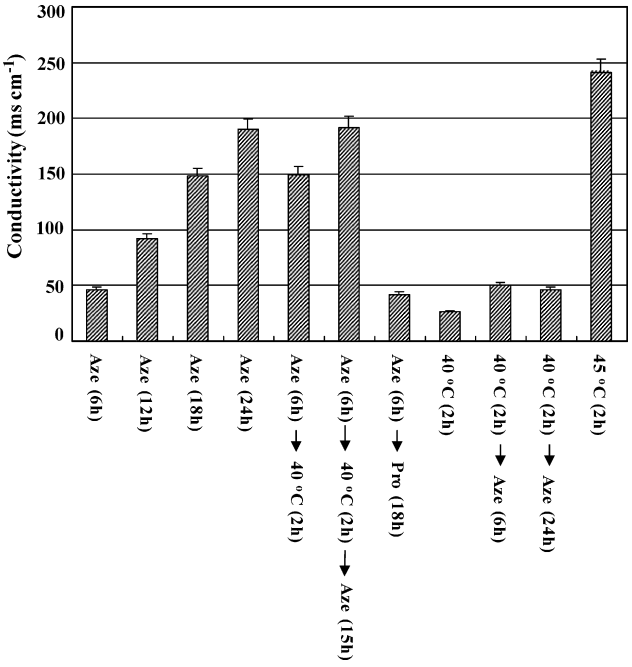

Fig. 4. Electrolyte leakage of soybean seedlings. Ten seedlings were treated with 10 mM Aze (28 °C), nonlethal HS (40 °C for 2 h) and lethal HS (45 °C for 2 h) as indicated. The conductivity of seedlings incubated in deionized water continuously at 28 °C was used as a blank. All values represent means ± S.D. of three separate experiments.

28 °C. After 6 h Aze treatment, the conductivity of the incubation medium began to increase (Fig. 4), thus causing injury to soybean seedlings. After 24 h Aze treatment, the electrolyte leakage was four-fold higher than that at 6 h. As well, 40 °C pre-incubation followed by 24 h Aze treatment effectively reduced the conductivity to about 30% of that with 24 h Aze treatment alone, whereas 6 h Aze treatment followed by nonlethal HS produced three-fold higher electrolyte leakage than nonlethal HS prior to 24 h Aze treatment (Fig. 4). The cellular leakage with 4 h 40 °C treatment following 6 h Aze treatment was higher than that with 2 h at 45 °C (data not shown). Furthermore, 10 mM proline could prevent the electrolyte leakage of 6 h Aze treatment. Replacing proline with other amino acids (e.g. Gly, Phe, Cys and Gln) following the 6 h Aze incubation did not rescue the leakage injury (data not shown). These results suggest that Aze can change the permeability of the plasma membrane, and the detrimental effect could be repressed by pre-accumulation of HSPs or proline.

4. Discussion

Amino acid analogs have been shown to induce HSP synthesis in both eukaryotic and prokaryotic cells [9,10,12,19,37,38]. The known association of nonlethal HS treatment with significant accumulation of HSPs was shown to confer thermotolerance in

antibodies. Molecular mass markers are shown on the left. (B) TTC staining of seedlings after nonlethal HS, Aze treatment, and lethal HS as indicated. (C) Three seedlings treated as in (B) were homogenized and extracted with 95% ethanol at 100 °C for 10 min. TTC reduction activity of seedlings was monitored by measuring the absorbance at 530 nm. All values represent means ± S.D. of three separate experiments.

soybean seedlings [21,24]. Aze can induce an HS-like response, the synthesis of HSPs, but Aze-treated seedlings do not tolerate lethal HS treatment [12], as one would expect for HS acclimation. In the current study, we further examined the Aze-induced HS-like response and the Aze effect in soybean seedlings. After being incorporated into proteins by replacing proline residues and resulting in the formation of misfolded proteins, Aze acted as a competent inducing signal in HS-like responses [11,36]. Of note, the level of HS mRNAs induced after 6 h Aze incubation lasted for more than 24 h, whereas the level declined within 6 h when Aze was replaced by proline (Fig. 1). These results indicate that Aze signaling is continuous on activation of HSP genes and is shut down in the presence of proline. However, after nonlethal HS treatment followed by Aze incubation, soybean seedlings acquired resistance to both thermal stress and Aze toxicity (Fig. 3), which suggests that the induction of HS response before Aze treatment conferred both tolerance to the injury effects of Aze and thermal stress in soybean seedlings. The data shown here are consistent with HSPs playing a pivotal role in conferring cross-tolerance to various forms of stress.

Plant sHSPs generally form multimeric protein complexes ranging from 200 to 310 kDa [30,33]. Accumulating evidence suggests that these proteins can function as molecular chaperones *in vitro* to prevent native protein from irreversible thermal aggregation and as a temporary reservoir of unfolded protein to facilitate refolding in the presence of other molecular chaperones [39,40]. Under HS, sHSPs are the most abundant proteins in plants, and the expression of an sHSP-CI in *E. coli* cells allows for survival under temperature stress [30,41]. *In vitro* results showed that the sHSP-CIs induced by Aze can form an oligomeric structure and suppress the aggregation of thermally denatured soluble proteins in soybean seedlings [12]. In this study, compared with 50 nM nonlethal HS-induced sHSP-CI complex, 50 nM Aze-induced sHSP-CI complex had less efficient suppression of CS thermal aggregation, whereas the mixture of 50 nM nonlethal HS- and 50 nM Aze-induced sHSP-CI complex, 100 nM Aze-induced sHSP-CI complex, and 100 nM nonlethal HS-induced sHSP-CI complex could act efficiently in preventing citrate synthase thermal aggregation (Fig. 2). Therefore, we confirm the observation that Aze-induced sHSP-CIs show chaperone activity *in vitro* although less efficient than that of nonlethal HS-induced sHSP-CIs. However, the effect of Aze on chaperone activity of sHSP-CIs needs further study.

Resistance to Aze has been proposed to occur by a number of mechanisms. Reduction of Aze uptake has been reported to prevent the toxic consequences of Aze treatment in one *Arabidopsis* mutant [35]. In yeast cells, Aze-resistant mutants result from mutation of the Gapl amino acid permease or expression of a unique acetyltransferase gene, *MPR1* [42,43]. As well, increased proline accumulation in *Saccharomyces cervisiae* provided cellular resistance to Aze toxicity [43]. Proline treatment is believed to compete with Aze for incorporation into proteins, resulting in alleviation of Aze inhibition on cellular growth [42]. The lethal effects found at 45 °C in soybean seedlings are in part caused by electrolyte leakage, but preincubation at 40 °C can repress this leakage [21]. HSP synthesis was found to preserve plasma membrane structure and repress solute leakage under the HS condition [44]. As well, membrane-sHSP associations have been shown to preserve membrane structure and integrity during thermal fluctuations [45]. Our results show that Aze-induced leakage of electrolytes at a nonlethal temperature is similar to that at a lethal temperature and injures the cellular membrane prior to accumulation of HSPs in the seedlings (Fig. 4). However, 2 h preincubation at 40 °C or 18 h proline incubation following 6 h Aze treatment at 28 °C ameliorated the leakage in soybean seedlings and conferred thermotolerance under lethal HS treatment (Fig. 4). This result coincides with the finding of Trent et al. [46], who showed that Aze treatment after HS did not cause membrane leakage in the hyperthermophilic archaeon *Sulfolobus shibatae*. Pre-accumulated HSPs not only mediate selective degradation of Aze-derived short-lived and abnormal proteins but also stabilize membrane integrity under Aze treatment [36]. In soybean seedlings, proline treatment alleviates the Aze effect and confers thermotolerance by elevating the distribution of HSPs in soluble protein [12], but the role of proline in preventing cellular leakage still remains unclear.

Taken together, our results show two observations from our *in vivo* and *in vitro* investigations. First, Aze treatment of soybean seedlings at 28 °C caused a continuous increase of electrolyte leakage and triggered the signal for induction of HS response. Thus, electrolyte leakage occurred prior to synthesis of sHSP-CIs, which function in thermoprotection of proteins *in vitro*. Second, plant HS response plays an important role in providing cross-tolerance for alleviating the detrimental effects of Aze treatment.

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