The chloroplast small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress

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Abstract The nuclear-encoded chloroplast-localized Hsp21 is an oligomeric heat shock protein (Hsp), belonging to the protein family of small Hsps and α -crystallins. We have investigated the effects of high temperature and oxidation treatments on the structural properties of Hsp21, both in purified recombinant form and in transgenic *Arabidopsis thaliana* plants engineered to constitutively overexpress Hsp21. A conformational change was observed for the 300 kDa oligomeric Hsp21 protein during moderate heat stress ($\leq 40^\circ\text{C}$) of *Arabidopsis* plants, as judged by a shift to lower mobility in non-denaturing electrophoresis. Similar changes in mobility were observed when purified recombinant Hsp21 protein was subjected to an oxidant. Exposure of Hsp21 protein to temperatures above 70°C led to irreversible aggregation, which was prevented in presence of the reductant dithiothreitol. The transgenic plants that constitutively overexpressed Hsp21 were more resistant to heat stress than were wildtype plants when the heat stress was imposed under high light conditions. These results suggest that the physiological role of Hsp21 involves a response to temperature-dependent oxidative stress.

INTRODUCTION

Many heat shock proteins (Hsps) are expressed constitutively, and act in different ways to prevent aggregation of unfolded proteins (Becker and Craig 1994; Hendrick and Hartl 1995; Ellis and Hartl 1996); a function which becomes increasingly important at high temperatures. Small heat-shock proteins (sHsps), belonging to the evolutionary conserved family of sHsps and α -crystallins (Vierling 1991; Caspers et al. 1995) are found in all eukaryotic organisms but are especially abundant in plants. Usually, sHsps are not expressed constitutively,

but only under heat stress conditions or at certain developmental stages. The sHsps are believed to play a role in thermotolerance (Neumann et al. 1989; Vierling 1991; Parsell and Lindquist 1993), but the molecular details of their functioning are unclear. Various studies have pointed to different targets for the thermoprotective role of sHsps, such as mRNA (Nover et al. 1989) and microfilament (actin) protection (Lavoie et al. 1993; Mehlen and Arrigo 1994). That sHsps can prevent heat-induced aggregation and facilitate renaturation of enzymes assayed in vitro (Jakob et al. 1993; Merck et al. 1993; Lee et al. 1995) suggests a chaperone function. For a sHsp that is localized in the cytosol of plant cells, it was recently shown that denatured substrate proteins were bound to the sHsp oligomers in vitro, presumably via hydrophobic regions that are exposed at increased temperatures, forming large complexes with up to one substrate protein bound per sHsp monomer (Lee et al. 1997).

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The chloroplast localized sHsp, Hsp21 (monomer size 21 kDa), is encoded by a nuclear gene and localized to plastids in root and leaf tissue (Vierling 1991). Hsp21 is usually not detectable in plants grown under optimal conditions, but it accumulates during high temperature stress. The protein is primarily found in the chloroplast stromal compartment, but severe stress treatments can drive it into an insoluble form (Chen et al. 1994; Osteryoung and Vierling 1994). It has been suggested that Hsp21 protects PSII during heat stress (Kloppstech et al. 1985; Heckathorn et al. 1998). An important structural feature of Hsp21 is a unique methionine-rich domain (amino acids 90–117), predicted to form an amphipathic α -helix, which is highly conserved among chloroplast sHsps from divergent plant species but not found in any other sHsp (Chen and Vierling 1991). A first insight into the structural organization of a sHsp was recently obtained when the structure of Hsp 16 from *Methanococcus jannaschii*, a hyperthermophilic archaeon, was resolved to atomic resolution (Kim et al. 1998). The *M. jannaschii* Hsp 16 forms a hollow spherical complex with an outer diameter of 130 Å, which is composed of 24 monomers. In each monomer, the C-terminal α -crystallin domain, common to all sHsps, is ordered in a composite β -sandwich fold, with one of the β -strands coming from a neighbouring monomer. Interestingly, if Hsp21 is organized in a corresponding arrangement, the methionine-rich conserved region is located in a disordered region of the structure, exposed either to the exterior or to the interior volume as a flexible arm, able to interact with yet unidentified target molecules.

We have previously purified the Hsp21 protein in oligomeric form, both from plant tissue and as recombinant protein (Härndahl et al. 1998; Suzuki et al. 1998) as a prelude to structural studies, in which, so far, small crystals have been obtained (Härndahl et al., unpublished results). During the course of our work, we have repeatedly seen that the stability of the Hsp21 oligomer is profoundly affected by oxidation, prompting us to investigate the response of the protein to oxidative conditions. In this paper, we describe changes in the Hsp21 oligomer detected both *in vivo* and *in vitro*, providing evidence that Hsp21 conformation is altered by oxidative stress. We also show that Hsp21 overexpression in transgenic plants confers a protective effect during exposure to combined heat and light stress, i.e. conditions that most likely involve oxidative stress. Our results support a role for Hsp21 in protection of chloroplasts from oxidative damage.

MATERIALS AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* were sown on soil and grown in a glass house with a supplementary light period

of 16 h at a photon flux density (PFD) of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$. After 2 weeks they were replanted to give four plants per pot. *Arabidopsis* plants that were 21 days old were used in experiments.

Vector construction, plant transformation and establishment of transgenic lines

To generate transgenic *Arabidopsis thaliana* plants (ecotype RLD) that constitutively overexpress Hsp21, a fragment corresponding to nucleotides 588 to 1609 of an Hsp21 genomic clone (GenBank accession # M94455) was amplified by polymerase chain reaction and subcloned behind the cauliflower mosaic virus 35S promoter into the plant transformation vector pMON530 (Rogers et al. 1987). Introduction of the transgene into *Arabidopsis*, selection of primary transformants, and genetic analysis of progeny were carried out as described previously (Osteryoung and Vierling 1992). In the Hsp21 overexpression line used in this study (5yf 6–4), the kanamycin resistance marker segregated as a single locus, and homozygous progeny were used in all experiments. The wild type control plants (5yai) were azygous progeny derived from the same parent line as the homozygous Hsp21 overexpressors.

Heat stress treatment

The plants were heat stressed in an Hereaus Vötsch climate-chamber, with 85% humidity to keep transpiration low, temperature 40°C and light intensity as stated in the figure legends. When plants were exposed to repeated heat stress for several days, the chamber was programmed in the following way: each day, the temperature was increased from 20 to 40°C during 1 h, kept at 40°C for 4 h and decreased to 20°C during 1 h. The light regime was either HL (high light; total light period 12 h, light intensity (PFD) $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the 4 h at 40°C , the light was gradually increased, and decreased during 4 h, respectively, between zero and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$) or LL (low light: total light period 8 h; the light was gradually increased, and decreased, during 4 h, respectively, between zero and $500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Recovery from a 4 h heat stress was performed at 20°C and PFD $120 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Detection of Hsp21 in *Arabidopsis* plants by immunoblotting

Leaves from four plants were withdrawn at different time-points and directly homogenized in a mortar. The homogenate was filtrated to remove cellular debris and the filtrate stored in liquid nitrogen and thawed before electrophoresis. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or

non-denaturing PAGE (see below), blotting transfer and detection of protein bands by polyclonal antibodies against Hsp21 were performed as described in Härndahl et al. (1998). As primary antibody, a polyclonal antiserum raised against recombinant *Arabidopsis* pre-protein, expressed in *Escherichia coli* with plasmid PJC20 (Clos and Brandau 1994), was used at 1:1000 or 1:500 dilution. As secondary antibody, the GAR-AP kit (Bio-Rad, Sweden) was used in which the conjugate alkaline phosphatase activity was visualized by a colour reaction.

Recombinant *Arabidopsis* Hsp21

Recombinant *Arabidopsis* Hsp21 was purified in 300 kDa oligomeric form, essentially as described earlier for recombinant pea protein (Härndahl et al. 1998), however it eluted somewhat earlier from the anionic exchange column (at 0.2 instead of 0.3 M NaCl). Purified recombinant *Arabidopsis* protein and pea protein were treated at different temperatures with additives, as indicated in the Figure legends. Samples were precipitated with acetone (200 μ l sample to 1 ml freeze-cold acetone) before electrophoretic analysis.

SDS-PAGE and non-denaturing PAGE

Electrophoresis of Hsp21 protein oligomers by non-denaturing PAGE was carried out as described earlier (Härndahl et al. 1998). The non-denaturing PAGE samples were mixed on ice with an equal volume of double strength sample buffer (see below). Acetone-precipitated samples were dissolved in sample buffer (0.125 M

Tris/HCl, 10% (v/v) glycerol), pH 7.8 and run in a buffer system without SDS. For SDS-PAGE the samples were mixed with an equal volume of solubilizing buffer (0.25 M Tris/HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 57.8 mM bromophenol blue), pH 6.8 and incubated at 70°C for 5 min.

Dry weight per plant determination

Plants were harvested and washed with distilled water. The roots were cut off and the plants were dried with paper. Each individual plant was then placed in a scintillation glass vial in an oven at 100°C for 48 h. Each plant, plus glass vial, was weighed before and after the drying treatment in the oven.

Chlorophyll fluorescence measurements

Leaves from *Arabidopsis* wildtype and overexpressing plants were selected randomly and were dark-adapted for 15 min. Leaves were placed on moist filter paper in a custom made holder. Chlorophyll fluorescence at room temperature was measured using a pulse-amplitude modulation fluorimeter (Model PAM 101/103; Walz, Effeltrich, Germany), and the maximum potential quantum yield of PSII (F_v/F_m) was determined from the mean values of at least five leaves. The maximum fluorescence yield was obtained by using a 800 ms saturation pulse of 2000 μ mol $m^{-2} s^{-1}$. The variable chlorophyll fluorescence ration F_v/F_m is linearly correlated with the number of functional PSII reaction centers (van Kooten and Snel 1990; Oquist et al. 1992).

Fig. 1 Oligomeric form of Hsp21 detected in wildtype (A) and overexpressing (B) *Arabidopsis* plants by immunoblotting after non-denaturing PAGE. C designates control plants before heat stress; 1 h, 2 h, 4 h designates samples withdrawn during the 4 h heat stress treatment at 40°C; 24 h, 48 h designates samples withdrawn during recovery at 20°C. The light intensity during the 1 day heat treatment was constant (PFD 500 μ mol $m^{-2} s^{-1}$). Asterisks indicate the Hsp21 oligomer upper band during heat stress (1, 2, 4 h), which in samples from wildtype plants is barely visible.

RESULTS

1. The Hsp21 protein oligomer undergoes a conformational change during heat stress of *Arabidopsis* plants

The content of Hsp21 in wildtype and transgenic *Arabidopsis* plants that constitutively overexpress Hsp21 has been analysed by SDS-PAGE and immunoblotting, by which the Hsp21 monomer can be detected as a 21 kDa band. In wildtype plants, Hsp21 could only be detected following heat stress, while in the transgenic plants, Hsp21 was expressed in both the absence and presence of prior heat stress, as has been described previously (Osteryoung and Vierling 1992; Osteryoung et al. 1994). The transgene was somewhat unevenly expressed among the individual plants, possibly due to gene silencing commonly observed in transgenic plant experiments (Matzke and Matzke 1998). In the transgenic line used in this study, approximately 25% expressed very little Hsp21 (data not shown). To compensate for this variation, a large number of plants were analysed in all experiments. Because transgene expression was low in some individuals, the phenotypic traits described below probably underestimate the actual effects of Hsp21 overexpression.

In both wildtype and transgenic plants, Hsp21 also can be detected in its native oligomeric state, as a 300 kDa band when analysed by non-denaturing PAGE and immunoblotting, as shown in Figure 1. To follow the Hsp21 oligomer during heat stress treatment, *Arabidopsis* leaves were sampled before, during and after heat stress. In wildtype and transgenic plants, the 300 kDa oligomer was readily seen 1–2 days after heat stress (Fig. 1A and 1B, recovery lanes). In the transgenic plants that overexpress Hsp21 constitutively, the Hsp21 band was also observed in the absence of heat stress (Fig. 1B, control lane). However, in samples that were withdrawn *during the 40°C treatment*, the appearance of Hsp21 was different. The 300 kDa band was barely detectable in the wildtype plants (Fig. 1A, heat stress lanes). Instead, a very weak band slightly above the 300 kDa position (marked with asterisks) was observed. In the case of the overexpressing plants, two bands became distinguishable during the 40°C treatment: one upper and one lower band at approximately 300 kDa. The appearance of the material in these lanes also had a somewhat blurred and indistinct appearance (Fig. 1B, Heat stress lanes). After the heat stress treatment, the Hsp21 oligomer was stable in the *Arabidopsis* plants for several days, and 5 days after heat stress, the amount of the Hsp21 oligomer in *Arabidopsis* declined (data not shown), consistent with the 52 h half-life estimated previously for Hsp21 in pea (*Pisum sativum*) plants (Chen et al. 1990).

Fig. 2 Oligomeric form of Hsp21 in wildtype and overexpressing *Arabidopsis* plants detected at Day 1 and Day 6 during a week-long heat stress treatment by immunoblotting after non-denaturing PAGE. Oex plants = overexpressing plants. C designates samples withdrawn before heat stress, either on Day 1 and 6; H designates samples withdrawn after 4 h heat stress, either on Day 1 or 6, during week-long daily heat stress treatment at 40°C, as described in Materials and methods for HL, except that maximum light intensity was set to PFD 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

The changes in Hsp21 electrophoretic mobility described above occurred during application of a single 4 h heat stress treatment. When the heat stress was repeated daily for 6 days, the appearance of the Hsp21 oligomer was dramatically different. Under these conditions, the oligomeric form could not be readily detected. Instead, antigenic material was blurred and smeared out in the whole lane, irrespective of whether the sample was withdrawn before or during the heat stress (Fig. 2, compare with Fig. 1 where only samples withdrawn *during* the heat stress were smeared). That Hsp21 was still present in these samples was confirmed by SDS-PAGE and immunoblot analysis (data not shown).

2. The purified *Arabidopsis* Hsp21 oligomer undergoes similar conformational changes

We have previously purified recombinant Hsp21 from both *Arabidopsis* and pea (Hårdahl et al. 1998; Suzuki et al. 1998). Initially we found that pea Hsp21, in contrast with the *Arabidopsis* protein, was difficult to resolve as discrete bands by non-denaturing PAGE, resembling the lack of discrete bands in Figure 2 in which the *Arabidopsis* leaves had been exposed to repeated heat stress over 6 days. However, further work showed that the addition of dithiothreitol (DTT) markedly improved resolution of the pea Hsp21 oligomer. Therefore, a closer investigation of the behaviour of the purified recombinant *Arabidopsis* Hsp21 oligomer was undertaken, in

Fig. 3 Purified *Arabidopsis* Hsp21 oligomeric protein after incubation at various temperatures in the absence (A) and presence (B) of DTT as visualized by non-denaturing PAGE. Purified *Arabidopsis* recombinant protein (0.05 mg/ml) was exposed to a step-wise increase in temperature (25–90°C), in absence (A) or presence (B) of 15 mM DTT, at a rate of 1°C per min, and, subsequently, the temperature was decreased in the same way (90–25°C). Samples were withdrawn for non-denaturing PAGE analysis at each step. (A) Samples incubated in absence of 15 mM DTT: Lane 1, control protein sample. Lanes 2–9, protein samples withdrawn during the temperature increase at 25, 30, 40, 50, 60, 70, 80, 90°C. Lanes 10–16, protein samples that were withdrawn during the subsequent temperature decrease at 80, 70, 60, 50, 40, 30, 25°C. (B) Samples incubated in presence of 15 mM DTT: Lanes 1–8, protein samples withdrawn during the temperature increase at 25, 30, 40, 50, 60, 70, 80, 90°C. Lanes 9–15, protein samples that were withdrawn during the subsequent temperature decrease at 80, 70, 60, 50, 40, 30, 25°C. Protein samples were precipitated with acetone, resuspended in sample buffer and loaded, 10 µg protein per lane. Proteins were detected by silver staining.

Fig. 4 Effect of various concentrations of H_2O_2 on purified *Arabidopsis* Hsp21 oligomeric protein as visualized by non-denaturing PAGE. All samples were H_2O_2 -treated for 1 h at 37°C at a concentration of 0.05 mg protein/ml. Protein samples were precipitated with acetone, resuspended in sample buffer and loaded, 10 µg protein per lane. Silver-staining allows detection of a set of very sharp, regularly spaced bands further down in the lanes in the *Arabidopsis* protein samples. This could reflect resolved dimers, tetramers, hexamers and octamers of Hsp21.

which the conditions of temperature and oxidation state were varied with the aim of better understanding the alterations in the Hsp21 oligomer observed during heat stress in vivo.

Purified recombinant *Arabidopsis* Hsp21 oligomer was exposed to a step-wise increase in temperature (25–90°C) at a rate of 1°C/min. Subsequently, the temperature was decreased in the same way (90–25°C). Samples were withdrawn for non-denaturing PAGE analysis at each 10°C-interval. The *Arabidopsis* Hsp21 oligomer remained essentially intact up to 70°C (Fig. 3A, lanes 1–7), but began to aggregate at higher temperatures, as demonstrated by the smeared appearance in the lane above the 300 kDa band. When the temperature was decreased, the discrete Hsp21 band was not regained, indicating irreversible aggregation of the protein. In contrast, aggregation above 70°C was largely prevented when the same step-wise temperature increase–decrease procedure was performed in presence of a reductant (15 mM DTT; Fig. 3B). Instead, the Hsp21 oligomer behaved as a sharp 300 kDa band throughout the whole treatment, irrespective of temperature.

The data shown in Figure. 3 clearly indicate that the recombinant Hsp21 oligomer is influenced by oxidative conditions. Therefore, the effect of the oxidant hydrogen peroxide, H_2O_2 , on the electrophoretic behaviour of the recombinant Hsp21 oligomer was investigated. Treatment *Arabidopsis* Hsp21 with 1 or 2 mM H_2O_2 showed only a small effect on the oligomer (Fig. 4, lanes 2–5). At 3 mM H_2O_2 , a shift to a lower mobility became apparent, and at higher concentrations the Hsp21 oligomer band became gradually more blurred and

Fig. 5 Wildtype and Hsp21-overexpressing *Arabidopsis* plants after 1 week of heat stress in high light. Overexpressing plants (to the right) are larger than wildtype plants (to the left) after combined heat and oxidative stress, whereas the corresponding plants that were not heat stressed (background) show no visible difference between wildtype and overexpressing plants. Heat stress was applied to 21 days old plants and continued for 1 week (each day 4 h at 40°C).

indistinct (Fig. 4, lanes 6–8). Above 10 mM H_2O_2 , the 300 kDa band was totally blurred and virtually undetectable (data not shown). The purified recombinant pea Hsp21 protein was even more sensitive to oxidation: the oligomer could barely be resolved as a distinct band even at low H_2O_2 concentrations (below 0.75 mM), and above 1 mM H_2O_2 this very diffuse and indistinct band virtually disappeared (data not shown). Taken together, these data show: 1) that low concentrations of oxidant cause a shift in mobility of the purified recombinant *Arabidopsis* Hsp21 that mimics that seen in heat-stressed *Arabidopsis* leaves (Fig. 1); 2) that purified recombinant Hsp21 from both *Arabidopsis* and pea undergoes oxidation-dependent loss of structural distinctness resembling that observed in *Arabidopsis* leaves exposed to repeated heat stress for 6 days (Fig. 2); and 3) that pea Hsp21 protein seems to be more sensitive than *Arabidopsis* Hsp21 to oxidative conditions.

3. Effect of Hsp21 overexpression in heat-stressed *Arabidopsis* plants depends on light conditions

In the field, high temperature conditions are frequently accompanied by high light, which together give rise to an oxidative environment in the chloroplast. This is because carbon dioxide fixation reactions decline during heat stress, and if incident light energy exceeds the consumption of energy by carbon assimilation, several active oxygen species are formed, such as hydrogen peroxide, superoxide anions, hydroxyl ions and singlet oxygen (Asada 1996). Consequently, heat stress and oxidative stress are often coincident in nature.

To begin investigating the physiological role of Hsp21 during combined heat and oxidative stress *in vivo*, the transgenic plants that overexpress Hsp21 and the corresponding wildtype were subjected to a daily heat stress for 7 days under either a high or low light regime. Under the high light regime, a visible difference between the transgenic and wildtype plants became evident following this 7 day treatment. The Hsp21 overexpressors appeared significantly larger and more vigorous than the wildtypes (Fig. 5). No such difference in the appearance of the plants was observed between transgenics and wildtypes after the 7 day heat stress treatment in low light (data not shown). To quantify these treatment effects, the dry weight per plant (DW/plant) was determined immediately following the 7 day treatment. Both heat stress treatments had a pronounced negative impact on plant productivity, since all the DW/plant values were much lower compared with corresponding control plants that had not been heat-stressed during the 7 days (Table 1). In high light, for the Hsp21-overexpressing plants, the DW/plant was 53% of unstressed control plants, whereas for the wildtype plants the DW/plant was 30% of unstressed controls. The differences between the transgenics and wildtypes was much less pronounced when the heat stress was imposed under low light. These results indicate that Hsp21 overexpression conferred a protective effect on the dry weight during combined heat and light (oxidative) stress.

To examine whether the heat stress treatments directly affected Photosystem II (PSII), F_v/F_m (variable chlorophyll fluorescence, an indicator of PSII capacity; van Kooten

Table 1 Dry weight per plant determined after heat stress in low and high light for wildtype and transgenic Hsp21-overexpressing *Arabidopsis* plants

	Control		Heat stress/low light		Heat stress/high light	
	DW/plant (mg)	% of control	DW/plant (mg)	% of control	DW/plant (mg)	% of control
Wildtype	^a 21.1 (1.3)	100%	^c 6.4 (0.4)	30%	^e 6.4 (1.6)	30%
Transgenic	^b 16.1 (1.0)	100%	^c 6.1 (0.3)	38%	^f 8.5 (2.5)	53%

Values within parenthesis are SEM. ^{a–f}: number of plants: ^a=102, ^b=100, ^c=55, ^d=56, ^e=84, ^f=86.

Table 2 Chlorophyll fluorescence (F_v/F_m) after heat stress in low and high light for wildtype and transgenic Hsp21-overexpressing *Arabidopsis* plants

	Control		Heat stress/low light		Heat stress/high light	
	F_v/F_m	% of control	F_v/F_m	% of control	F_v/F_m	% of control
Wildtype	0.79 (0.045)	100%	0.71 (0.006)	90%	0.73 (0.007)	92%
Transgenic	0.70 (0.050)	100%	0.74 (0.007)	95%	0.75 (0.003)	95%

Values within parenthesis are SEM.

and Snel 1990; Oquist et al. 1992) was measured, and the values are shown in Table 2. Only very minor changes in F_v/F_m were observed in response to the 7 day heat stress under either low or high light, and there was little difference between the transgenic and wildtype plants. Heat stress at higher temperatures than those used in this study can lead to a decline in F_v/F_m to half the control value or less (Yamane et al. 1997; Miyao-Tokutomi et al. 1998).

DISCUSSION

Conformational changes due to oxidation of the Hsp21 oligomer

Could the shift in Hsp21 mobility (Fig. 1), and the disintegration of the Hsp21 oligomer (Fig. 2) seen in heat-stressed *Arabidopsis* plants be caused by oxidation? Our observation that similar transitions can be evoked in the purified recombinant Hsp21 oligomer by an oxidant (Fig. 4) suggest that this is so. The temperature-dependent aggregation (Fig. 3) also seems to be promoted by oxidation, or rather, to be prevented by an added reductant. The tendency for aggregation is common to sHsps as well as α -crystallins, a phenomenon occurring in cataract aged lenses and in plaque deposits in neurodegenerative diseases (Groenen et al. 1994). In both cases oxidative modifications in the proteins have been detected. The data presented in this paper indicate that an oxidation-dependent conformational change (shift from lower to upper band; Fig. 4) in the Hsp21 protein oligomer could precede such aggregation.

The pronounced smearing of Hsp21 seen in Figure 2 after repeated heat stress in *Arabidopsis* is similar to that described previously for heat stressed pea plants (Chen et al. 1994; Osteryoung and Vierling 1994). In those studies, the smearing was correlated with a temperature-dependent redistribution of the Hsp21 protein from soluble to insoluble form. Such insolubility of Hsp21, and the related inability to form sharp, distinct bands on non-denaturing PAGE, could be caused by extensive, secondary oxidative modifications in Hsp21, whereas the oxidation-dependent shifts into lower mobility of the

Hsp21 oligomer could be caused by some primary, more specific amino acid oxidation.

Oxidation of conserved methionine residues

The unique methionine-rich region, which is highly conserved among chloroplast sHsps from divergent plant species but not found in any other sHsp and predicted to form an amphipathic α -helix (Chen and Vierling 1991), could have a specific role in this matter. We have found that methionine sulfoxidation is caused by the same H_2O_2 concentrations which cause the mobility shift in Figure 1 and 4 (Gustavsson et al. 1998). Further investigations by mass spectrometry extend the previous data, and point to a reversibility of the sulfoxidation (Gustavsson et al., in preparation). Methionines are the most readily oxidized amino acid residues (Vogt 1995), and several recent papers suggest an importance of methionine sulfoxidation in cells (Levine et al. 1996; Chao et al. 1997), maybe as a primitive antioxidant system (Levine et al. 1996). Other amino acid residues that can become oxidized under more severely oxidizing conditions, and cause the smeared appearance in Figure 2 and 4, are cysteine, tyrosine, tryptophan and histidine residues, and there are also other post-translational modifications of oxidative nature, such as asparagine deamidation. Oxidation of cysteines may cause improper disulfide bond formation that can lower the denaturation temperature of a protein (Senisterra et al. 1997). Whereas the denaturation temperature and conformational stability is increased by native unstrained intramolecular disulfide bonds, non-native disulfide bonds increase the strain energy, thereby causing a destabilization of the folded polypeptide (Freeman et al. 1995). The probabilities for non-native destabilizing disulfide bonds to form is higher the higher the number of cysteine residues, and could explain the larger sensitivity to oxidation for the pea Hsp21 protein since the pea Hsp21 has three cysteine residues per monomer, compared to only one per monomer for the *Arabidopsis* Hsp21.

Does Hsp21 oxidation play a role in vivo?

The Hsp21-overexpressing transgenic *Arabidopsis* plants have been of great importance for this study. The large

amounts of Hsp21 protein in the transgenics allowed the detection of the upper Hsp21 band during heat stress (Fig. 1B) that was difficult to see in wildtype plants (Fig. 1A), and they also allowed an evaluation of the effect of excess Hsp21 for plant performance under different types of heat stress (Table 1). To further investigate the importance of Hsp21 in vivo, we have also tried to raise transgenic antisense plants. So far, we have either obtained plants with only moderately reduced Hsp21 level that show no phenotypic difference from wildtype, or plants with no Hsp21 that grow very poorly, even in absence of heat stress (unpublished results). These observations may indicate an important role for Hsp21 in unstressed plants, maybe during certain developmental stages, such that decreased viability results from its absence as observed for Hsp60-antisensed plants (Zabaleta et al. 1994). Earlier data indeed have reported that Hsp21 could be detected in very young barley plants in absence of heat stress (Kruse et al. 1993).

At the heat stress temperatures employed in our experiments, we found no evidence for damage to PSII. Decreased plant performance at high temperatures despite the apparent lack of damage to PSII is consistent with accumulating evidence that carboxyfixation is reduced by heat stress at a lower threshold value than PSII (Kobza and Edwards 1987), and that damaging effects on PSII occur above, but not below, 40°C (Feller et al. 1998). Thus, the increased temperature tolerance correlated with Hsp21-overexpression might be explained by protection of the carbon dioxide-fixation reactions, either directly or indirectly, possibly by protection of the heat-sensitive key component, Rubisco activase (Feller et al. 1998). The transgenic Hsp21-overexpressing *Arabidopsis* plants will be valuable tools in further addressing this question.

Do the transgenic Hsp21-overexpressing plants provide evidence that Hsp21 protects against oxidative stress? The data on DW/plant demonstrating improved performance in the Hsp21-overexpressing plants during heat stress in high light (Fig. 5, Table 1) provide preliminary support for this idea. Similar results were reported recently for transgenic Hsp21-overexpressing tobacco, which also resisted heat stress better than wildtypes in a light-dependent manner (Miyao-Tokutomi et al. 1998). High light during heat stress, as compared with low light, is predicted to have two opposing effects: on one hand, heat stress is better tolerated if the light is sufficiently high to maintain high rates of the photosynthetic light reactions (Schreiber and Berry 1977); on the other hand the level of oxidative stress is increased. The Hsp21-overexpressing *Arabidopsis* plants could be better equipped to deal with heat and oxidative stress than wildtype plants due to an Hsp21-related oxygen

scavenger function, added to its presumed chaperone function.

Role of sHsps in oxidative stress

In mammalian cells, accumulating data demonstrate a strong coupling between sHsps and oxidative stress, reviewed in (Arrigo 1998). This phenomenon is linked to an ability of sHsps to counteract not only heat stress, but also other types of stress, like oxidative stress, TNF α (tumour necrosis factor)-induced cytotoxicity and toxic chemicals used in cancer chemotherapy. In all these cases, sHsps have been shown to decrease the intracellular level of reactive oxygen species in a glutathione-dependent way (Mehlen et al. 1996a). In a similar way the sHsp expression even appears to be essential in preventing differentiating embryonic stem cells from undergoing apoptosis, i.e. programmed cell death (Mehlen et al. 1996b; Mehlen et al. 1997a). The underlying molecular mechanisms whereby sHsps decrease reactive oxygen species and increase glutathione levels are as yet unknown. The tripeptide glutathione (Glu-Cys-Gly) acts as a redox buffer in cells, and this novel regulatory role of sHsps may involve chaperoning of enzymes involved in the pathways of glutathione formation or reactive oxygen species metabolism, by translational regulation, or by degradation of oxidized proteins (Arrigo 1998). For human carcinoma and HeLa cells, structural and conformational changes have been described for the sHsp (Hsp27) oligomer, which are important for the protective role of Hsp27, and for the modulation of the glutathione levels (Mehlen et al. 1995a; Mehlen et al. 1995b; Mehlen et al. 1997b).

It is yet unclear how the changes in the oligomeric structure of mammalian Hsp27 relate to the structural and conformational changes that we have described in this paper for the Hsp21 oligomer. At present, we are investigating more closely the thermodynamic stability of the recombinant *Arabidopsis* Hsp21 oligomer in different oxidative states (Hårdahl et al., in preparation). Further investigation is also needed to clarify if and how Hsp21 influences glutathione levels in *Arabidopsis*, and what role the conserved methionine-residues play, for the conformational change in the Hsp21 oligomer and for the protective effect. In any event, the chloroplast sHsp may offer important new insights into the molecular mechanisms of sHsps in oxidative stress. The oxygen-producing environment in the chloroplast presumably have made the Hsp21 extremely well adapted to handle reactive oxygen species, maybe to an even larger degree than sHsps in mitochondria, which already are known to play a key role in programmed cell death (Polla et al. 1996). For further studies, the experimental system offered by *Arabidopsis* has several advantages, allowing

mapping of pathways in an eukaryotic organism by molecular genetics and gene knock-out (Meinke et al. 1998), in parallel with protein studies on recombinant protein, including site-directed mutagenesis.

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