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The H1 histone variant of tomato, H1-S, is targeted to the nucleus and accumulates in chromatin in response to water-deficit stress

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Abstract. Water deficit has a significant impact on patterns of gene expression. Based on the deduced amino acid sequence, it has been proposed that the drought and abscisic acid-induced gene (*his1-s*) of tomato (*Lycopersicon esculentum* Mill.) encodes an H1 histone variant. To study the role of H1-S it is important to understand the expression characteristics of the protein. To identify the *his1-s* product in vivo the *his1-s* cDNA was fused to a $(His)_6$ tag and overexpressed in *Escherichia coli*. The H1-S fusion protein was used to generate an antibody that recognized a protein with an apparent molecular weight of 31 kDa that accumulates in response to water deficit in the whole plant and detached leaves. A time course of *his1-s* expression showed that protein accumulation is delayed compared to the mRNA accumulation in both the whole plant and detached leaves. Cellular fractionation, immunofluorescence and H1-S::β-glucuronidase fusion analyses in transgenic tissues were used to determine the cellular localization of H1-S. The results showed that H1-S accumulates in nuclei and is associated with chromatin of wilted tomato leaves. The drought- and abscisic acid-induced gene *his1-s* encodes a linker-histone subtype specifically accumulated in the nuclei and chromatin of tomato leaves subjected to water-deficit conditions. Although the molecular mechanism of H1-S function is still unclear, the expression characteristics of H1-S are consistent with a potential role of this protein in the regulation of gene expression in response to water deficit.

Key words: Chromatin – H1 histone – *Lycopersicon* (water deficit) – Water deficit

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Abbreviations: ABA = *cis*-abscisic acid; DAPI = 4′-6′-diamidino-2 phenylindole; GUS = β-glucuronidase

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Introduction

It is well known that plant responses to water deficit promote changes in gene expression. Many water-deficit-induced genes have been isolated, and the DNA sequence determined over the last two decades (Bray 1997). Most of the current knowledge of the possible role of these genes has been inferred from the deduced amino acid sequence with little support from empirical evidence. Functional inferences have been derived from gene-expression studies, mainly at the messenger RNA level, with little knowledge about the accumulation and expression characteristics of the proteins encoded by the water-deficit-induced genes. In tomato (*Lycopersicon esculentum*), a drought- and abscisic acid (ABA)-induced gene *his1-s* was isolated and the DNA sequence determined (Bray et al. 1999). The deduced amino acid sequence of this gene has strong homology with the central globular domain of H1 histone, and it was predicted that *his1-s* encodes a linker-histone variant (Bray et al. 1999). However, no empirical evidence was provided to support the conclusion that the product of *his1-s* is an H1 histone protein.

H1 histones or linker histones are abundant basic proteins that interact with linker DNA between nucleosome cores thus facilitating the compaction of chromatin (Wolffe et al. 1997). In metazoans, linker histones have a characteristic three-domain structure consisting of a central globular domain flanked by N- and C-terminal tail regions (Wolffe et al. 1997). The globular domain is composed of three α-helices with a structure similar to that of known transcription factors (Ramakrishnan et al. 1993). It has been proposed that the helices of the globular domain interact with the DNA at the dyad and the linker (Zhou et al. 1998) within the nucleosome, while the C-terminal tail binds the linker DNA, neutralizing its charge and facilitating chromatin condensation (Staynov and Crane Robinson 1988).

H1 histone genes have been cloned and sequenced from several plants such as tomato (Jayawardene and Riggs 1994), tobacco (Szekeres et al. 1995), pea (Gantt and Key 1987), maize (Razafimahatratra et al. 1991),

wheat (Yang et al. 1991), *Arabidopsis thaliana* (Gantt and Lenvick 1991; Ascenzi and Gantt 1997), and *Lycopersicon pennellii* (Wei and O'Connell 1996). It is well established that linker histones represent a family of isoprotein species (Cole 1984). In animals as well as in plants, H1 variants have been isolated from different organs, tissues, or during different stages of development. H1 subtypes mainly differ in the amino acid sequence and the length of the C-terminal tail, which affects the ability to mediate the formation of a higher-order chromatin structure (Cole 1984). The plant linker histones can presently be divided into two groups based on amino acid sequence (Wei and O'Connell 1996; Ascenzi and Gantt 1997; Bray et al. 1999).

It has been thought that linker histones have a structural role in chromatin organization and in the general repression of gene expression (Laybourn and Kadonaga 1991; Wolffe et al. 1997). More recently, it has been shown that linker histones have a specific role in repressing and activating particular genes (Shen and Gorovsky 1996). The expression of different subtypes during specific stages of development is related to the regulation of expression of specific genes (Bouvet et al. 1994). In this respect, the accumulation of a linker-histone variant might be required in the complex machinery of gene-expression regulation activated during the water-deficit response. If so, the product of the water-deficit- and ABA-induced gene, *his1-s*, has to be identified in vivo. Using a combination of approaches, the water-deficit-induced expression patterns of *his1-s* and the corresponding protein, and the subcellular location of H1-S have been studied. Our results indicate that the water-deficit-specific linker histone accumulates after a lag time compared to the mRNA and that the protein accumulates in the nucleus.

Materials and methods

Water-deficit treatments and physiological measurements. Leaf samples were collected from well-watered tomato plants (*Lycopersicon esculentum* Mill., cv. Ailsa Craig) and wilted at room temperature to 88% of their original fresh weight, then incubated in plastic bags for 24 h. As a control, the petioles of detached leaves were placed in water for 24 h. For the whole-plant stress, 6- to 7-week-old tomato plants (cv. Ailsa Craig) were not watered for a period of 15 d. Plants of approximately equal size, maintained in a well-watered condition, were used as a control. Leaf conductance, relative water content (RWC) and ABA content were measured as described in Griffiths and Bray (1996).

Antibody production. In order to identify the product of *his1-s* in vivo, an antibody was raised in rabbit by ligating the *Eco*RI/*Hind*III fragment (encoding 142 amino acids of the C-terminal region) of the pLE20 cDNA to a $(His)_6$ tag in the pTrcHisC vector (Invitrogen Corporation, San Diego, Calif., USA). The protein was overexpressed in *E. coli* and was purified on a nickel-affinity column under denaturing conditions (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4.0; Invitrogen). Eluted samples were dialyzed against 0.1 mM Tris-HCl, 0.1% Triton X-100, pH 8.0. The His-tagged protein was further purified by SDS-PAGE using a 10% polyacrylamide gel, followed by staining with 0.3 M $CuCl_2$ and excision of the relevant molecular weight band from the gel (Harlow and Lane 1988). Following dialysis against 0.1 mM Tris-HCl (pH 8.0), samples were concentrated in Centricon units (Amicon Corp., Bedford, Mass., USA). Recombinant protein samples were stored at −20 °C prior to immunization (Harlow and Lane 1988).

Isolation and analysis of RNA. Total RNA was extracted from 5 g of tomato leaflets using a LiCl-phenol extraction method (Prescott and Martin 1987). At specific time points, and along with samples for RWC, ABA and protein analysis, the youngest, fully expanded leaves were collected, immersed in liquid nitrogen, and stored at −80 °C. Thirty micrograms of total RNA was separated by size in formaldehyde denaturing 1.2% agarose gels (Sambrook et al. 1989), transferred to nylon membranes, cross-linked, prehybridized and hybridized according to Cohen and Bray (1990) with the following exceptions. Prior to prehybridization the membranes were washed for 2 h in a solution of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% sarcosyl, and 1 M NaCl at 42 °C to minimize non-specific hybridization. Polyadenylated RNA and tRNA were not added to the solutions and hybridization was carried out overnight at 65 °C. An aliquot of 1×10^7 cpm of ^{32}P-labeled RNA synthesized from pLE20 cDNA was placed in 15 ml of hybridization fluid. After hybridization, membranes were washed as follows: 5 min in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7), 0.2% sarcosyl at room temperature; two washes 20 min each at room temperature in 2× SSC, 0.2% sarcosyl; two washes 30 min each at 42 °C in 1× SSC, 0.2% sarcosyl; and two washes 30 min each at 65 °C in 0.1× SSC, 0.2% sarcosyl. Membranes were wrapped in plastic wrap and exposed to X-ray film at −80 °C.

Protein extraction. Isolation of tomato leaf histones was adapted from procedures previously described for barley leaves (Muller et al. 1980; Langenbuch et al. 1983). Approximately 15 g of leaf tissue was collected at the same time as samples for ABA and RNA analyses. Extraction and isolation steps were carried out at 4 °C. Plant material was ground to a fine powder in liquid nitrogen before being transferred to a beaker containing 200 ml of grinding medium [0.25 M sucrose, 10 mM Tris-base (pH 8.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 25 mM $NaHSO_3$, 0.1 mM phenylmethylsulfonyl fluoride]. The tissue was homogenized for 1 min at full speed using a Waring Blender, followed by filtration through eight layers of cheesecloth, and further filtration through nylon mesh (0.25 μm gauge). The filtrate was then dispensed into glass Corex tubes and centrifuged at 8,500 rpm (JS13.1 rotor, Beckman, Fullerton, Calif., USA) for 10 min. The pellet was resuspended in 2 ml of washing medium (grinding medium plus 0.5% Triton X-100) and re-centrifuged. The pellets were then resuspended in 2 ml of centrifugation medium [1.7 M sucrose, 10 mM Tris-base (pH 8.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 12 mM $NaHSO_3$, 0.1 mM phenylmethylsulfonyl fluoride]. In separate tubes (16 × 106 mm Polyallomer; Beckman), 9 ml of centrifugation medium was added, the resuspended pellets were overlaid, and centrifuged at 14,000 rpm (SW28 ultracentrifuge rotor, Beckman) for 1 h at 4 °C. The chromatin pellets were resuspended, using a glass rod, in 1 ml dilute saline citrate (15 mM NaCl, 1 mM sodium citrate, 20 mM EDTA) and combined. On ice, 1/4 volume of 1 N H_2SO_4 was added slowly with stirring for 30 min. The sample was transferred to a 30-ml Corex tube and centrifuged at 8,500 rpm (JS13.1 rotor, Beckman) for 30 min. The protein was precipitated from the supernatant at 4 °C overnight by the addition of 18% trichloroacetic acid. The protein was recovered by centrifugation at 8,500 rpm (JS13.1 rotor, Beckman) for 15 min. The pellet was washed in cold acetone and recentrifuged. The pellet was dried and resuspended in loading buffer without bromophenol blue [125 mM Tris-HCl (pH 6.8), 4% SDS, 40% glycerol, 0.1% 2-mercaptoethanol]. Protein concentrations were determined using the Peterson (1977) microassay.

Extraction of nuclei. Nuclei were extracted using a modified procedure from Luthe and Quatrano (1980). Approximately 15 g of tomato leaves were collected, quickly frozen in liquid nitrogen, ground to fine powder and transferred to the Omnimixer cup

containing cold Honda Buffer [0.44 M sucrose, 2.5% Ficoll 400 (Sigma, St. Louis, Mo., USA), 0.5% Dextran-T40 (Amersham Pharmacia BioTech, Piscataway, N.J., USA), 25 mM Tris-HCl (pH 8.5), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.5% Triton X-100]. The tissue was homogenized at top speed three times for 30 s then filtered through eight layers of cheesecloth, and through nylon mesh (0.25 μm gauge). Nuclei were pelleted at 5,000 rpm for 5 min using a JA20 rotor at 4 °C. The green pellet was resuspended in 5 ml cold Honda buffer and layered on pre-chilled Percoll gradients, formed in 30-ml Corex tubes with 5-ml layers: 2 M sucrose, 80%, 60%, 40% Percoll diluted in 10× gradient buffer [250 mM Tris-HCl (pH 8.5), 100 mM $MgCl_2$]. After centrifugation at 4,000 rpm (JS13.1 rotor, Beckman) for 30 min at 4 °C, nuclei were recovered at the interface between 80% Percoll and 2 M sucrose. Nuclei were washed twice by resuspending in 2 ml Honda buffer and centrifuging at 4,000 rpm (JS13.1 rotor, Beckman) for 10 min. The final pellet was checked for purity using the UV fluorescent microscope after staining with 1.5 mM DAPI (4′-6′-diamidino-2 phenylindole). Highly purified nuclei were resuspended in 2 ml of dilute saline citrate buffer. Acid-soluble proteins were extracted as above.

Total protein extraction. Proteins were isolated from approximately 15 g of leaf by the phenol extraction method essentially as described by Bray (1988). After extraction and centrifugation at 5,000 rpm (JS13.1 rotor, Beckman) for 5 min, the phenol phase was re-extracted. Five volumes of 0.77% ammonium acetate in methanol (w/v) were added and the proteins were precipitated overnight at −20 °C. After centrifugation at 10,000 rpm (JS13.1 rotor, Beckman) for 5 min at 4 °C, the pellet was washed twice with the ammonium acetate/methanol solution, once with cold acetone, resuspended in 4 ml of dilute saline citrate, and extracted for histones as above.

Gel electrophoresis, electroblotting and immunoblotting. Proteins were separated by SDS-PAGE according to Thomas and Kornberg (1975). Their protocol uses the basic system of Laemmli (1970) with three modifications: the concentration of Tris buffer in the resolving gel (18% polyacrylamide) is increased to 0.75 M, the ratio of acrylamide:bis-acrylamide is increased to 30:0.15, and the electrode buffer is composed of 0.05 M Tris, 0.38 M glycine, 0.1% SDS, pH 8.3. Samples prepared in 125 mM Tris-HCl (pH 6.8), 4% SDS, 40% glycerol, 0.1% 2-mercaptoethanol and 20 mg bromophenol blue were warmed to 65 °C for 5 min. Low-molecular-weight standards (Bio-Rad Laboratories, Hercules, Calif., USA) and 30 μg of proteins were separated (Protean II; Bio-Rad) and electroblotted onto nitrocellulose (Sambrook et al. 1989).

Immunoblotting was carried out at room temperature as follows: nitrocellulose was blocked for at least 2 h in TBST [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] with 5% (w/v) nonfat dried milk powder; rinsed once for 5 min in TBST alone; incubated 2 h with antiserum to H1-S diluted 1:2000 in TBST, 1% bovine serum albumin (BSA), and 2% goat serum albumin (GSA); given one brief rinse in TBST; washed (3 × 5 min) in TBST, 1% BSA, 1% GSA; incubated for 1 h with goat anti-rabbit IgG alkaline phosphatase conjugate diluted 1:3000 in TBST, 1% BSA, 1% GSA; and washed (3 × 5 min) in TBST. Detection of the secondary antibody was with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Immunolocalization by fluorescence and light microscopy. Leaflets were cut with a sharp razor blade into 2- to 3-mm sections. A section was placed on a thin layer of embedding compound (OCT; Miles Laboratories, Naperville, Ill., USA) applied to a cold sample holder, OCT was added to completely cover the sample holder, and it was immersed into freezing solution (Forane 22-chlorodifluoromethane and liquid N_2) for 5–10 s. Cryostat sections (4 μm) were made, placed on 0.01% poly-L-lysine-coated multi-well slides, and air-dried.

The cryosections were covered for 1 h at room temperature with 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20, 5 mM NaCl (TBS low salt), for the low-salt treatment, and 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20, 150 mM NaCl (TBS) for the control. Sections were fixed for 20 min at room temperature using 4% formaldehyde in TBS low salt or in TBS, and washed (3 × 5 min) with TBS low salt or TBS.

Immunolocalization on the cryosections was done using TBS low salt and TBS for the two different treatments as follows: sections were blocked overnight at 4 °C in TBS, 1% BSA, 1% GSA, 0.1% Tween 20; incubated for 1 h with antiserum to H1-S 1:100 in TBS, 1% BSA, 1% GSA, 0.1% Tween 20; washed (3 × 5 min) in TBS, 1% BSA, 1% GSA, 0.1% Tween 20; incubated for 1 h with monoclonal anti-rabbit IgG biotin conjugate antibody (Sigma) 1:1000 in TBS, 1% BSA, 1% GSA, 0.1% Tween 20; washed (3 × 5 min) in TBS, 1% BSA, 1% GSA, 0.1% Tween 20; incubated for 30 min with ExtrAvidin FITC (fluorescein isothiocynate, Sigma) conjugate 1:400 in TBS, in the dark; and washed (3 × 5 min) in TBS in the dark. Slides were counterstained with DAPI at 2 μg/ml and mounted in mounting medium for fluorescence microscopy (Vectashield H-1000; Vector Laboratories, Burlingame, Calif., USA). Negative controls for the primary antibody sections were treated for 1 h with pre-immune rabbit antiserum; as controls for the secondary antibody, some sections did not receive treatment with the primary antibody; as negative controls for the Extra-Avidin FITC conjugate, some sections were not treated with the biotinylated secondary antibody.

Construction of the H1-S::GUS fusion. The stop codon of *his1-s* was removed and mutagenized to a *Bam*HI restriction site by polymerase chain reaction (PCR), using the 5′ T3 primer (AATTAACCCTCACTAAAGGG) and the 3′ primer (CCATG-CGGATCCTGCAATTTTCTTGGCCC). The mutagenized PCR product (*Spe*I/*Bam*HI) was ligated into the plant transformation vector pBI221 (*Xba*I/*Bam*HI) (CLONTECH Laboratories, Palo Alto, Calif., USA) between the 35S cauliflower mosaic virus (CaMV) promoter and β-glucuronidase (GUS) creating a translational fusion. The resulting vector, pBI221H1-S::GUS, was used for transient expression assays in onion epidermis. To construct the fusion in the transformation vector pBI121 used for the *Agrobacterium*-mediated transformation of tomato leaf disks, the H1-S::GUS fusion was removed from pBI22I and ligated into pBI121 using the *Sst* I/*Hind* III restriction sites.

Transient expression in onion epidermis using microprojectile bombardment. Transient expression of pBI221H1-S::GUS or pBI221 in onion epidermal layers was according to Varagona et al. (1992). Each construct was analyzed 4 times. The pressure within the sample chamber was reduced to 635 mm Hg prior to bombardment. The tissues were bombarded with DNA-coated particles from a distance of 5 cm using high-velocity flow of He (2.5–3.0 kg/cm^2). After particle bombardment, Petri dishes were sealed with Parafilm and incubated overnight at 24 °C.

Construction of transgenic tomato plants and histochemical analyses. Transformation of tomato (cv. UC82b) with the H1-S::GUS fusion followed the procedure reported by Chao et al. (1999). Primary transformants were used for the reported experiments. The colorimetric X-gluc assay was used to detect GUS activity. Tissues were incubated at 37 °C in 50 mM $NaPO_4$ (pH 7.0), 1 mM EDTA, 0.001% Triton X-100, 10 mM 2-mercaptoethanol, 0.05 mM sodium ferricyanide and sodium ferrocyanide, containing 2 mM X-gluc. The blue color was detected after 1 h of incubation. Tissues mounted on glass slides using water were analyzed by standard microscopy (Zeiss). Images were recorded on Kodak Extachrome Gold.

Results

Accumulations of his1-s RNA and H1-S protein are temporally separated. Tomato leaves were detached,

wilted to 88% of their original fresh weight, collected after 2, 8, 12, and 24 h of water-deficit stress imposition, and analyzed for *his1-s* mRNA and protein levels, and ABA content (Fig. 1). Accumulation of *his1-s* mRNA was an early response to water-deficit; an increase in *his1-s* mRNA level was detected within 2 h of imposition of water deficit (Fig. 1B) and was at the highest level from 8 to 12 h. In acid-soluble protein extracts of chromatin, an increase in H1-S protein was first detected at 8 h of water deficit, and the highest level occurred from 12 to 24 h (Fig. 1A). The ABA content increased significantly after 2 h in detached leaves. A maximum increase of 7.6-fold compared to non-stressed leaves was achieved after 8 h of water deficit (Fig. 1C). The increase in ABA content occurred prior to the increase in *his1-s* mRNA which was followed by an elevation in the content of H1-S protein.

Whole tomato plants were subjected to water deficit to determine if the relative accumulation of protein and mRNA was related to whole-plant signaling events. The water status of whole plants was monitored during the period of water-deficit treatment (Fig. 2). Relative water content and stomatal conductance were determined every other day. The relative water content declined gradually throughout the experiment (Fig. 2A). Reductions in stomatal conductance were evident by day 3 in plants subjected to water deficit (Fig. 2B). Whole plants subjected to soil water deficit had an increase in bulk leaf ABA content of approximately 2-fold after 3 d compared to those remaining well-watered (Fig. 2C). By day 9, the ABA content of leaves of the plants that did not receive water had increased to the maximum, a 5-fold increase compared with the well-watered samples.

The expression pattern of *his1*-s was investigated at the mRNA and protein levels in leaves from whole plants that were subjected to soil water deficit. Non-stressed leaf samples were collected from well-watered plants and stressed leaf samples were collected after 1, 3, 9, and 15 d from the time water was withheld (Fig. 3). Leaves from well-watered plants exhibited low levels of

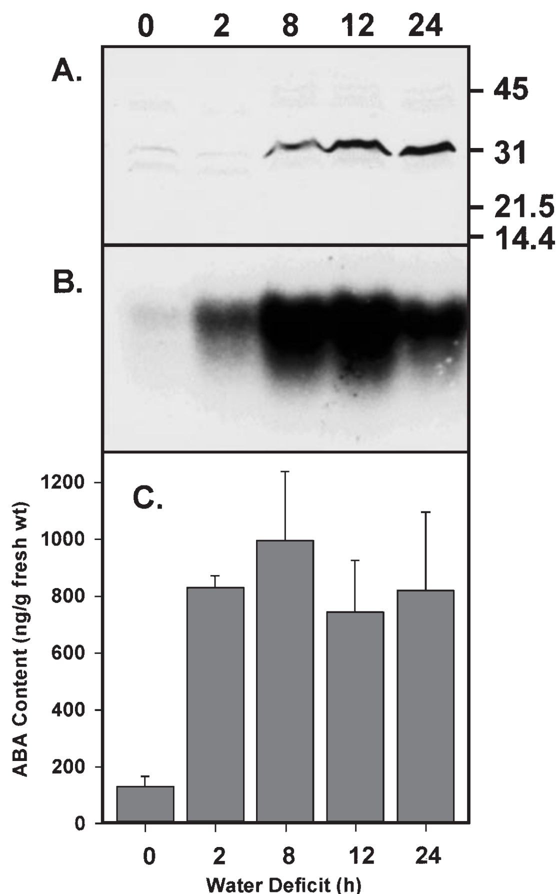

Fig. 1A–C. Expression of H1-S and its mRNA compared to ABA accumulation in detached tomato leaves subjected to water deficit. Tomato leaves were detached, wilted to 88% of their original fresh weight and sampled at 0, 2, 8, 12 and 24 h after the water deficit was initiated. **A** Chromatin preparations were prepared from leaves at each time, subjected to SDS-PAGE according to Thomas and Kornberg (1975) and immunoblotted. The protein was detected with antiserum to tomato H1-S. **B** RNA was extracted and separated by formaldehyde agarose gel electrophoresis, blotted and hybridized with the cDNA encoding H1-S. **C** ABA was extracted from leaves at each of the time points and quantitated by ABA radioimmunoassay

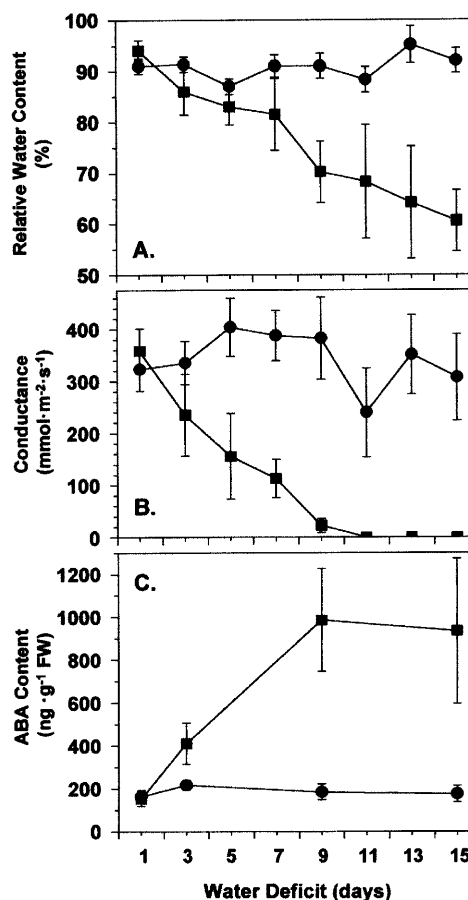

Fig. 2A–C. Whole tomato plants, 6–7 weeks old, were subjected to soil water deficit, and relative water content, stomatal conductance and ABA content were measured. Water was withheld from plants (■) or plants were fully watered (●) for 15 d. **A, B** Leaf relative water content (**A**) and stomatal conductance (**B**) were measured every other day. **C** Leaf ABA content was determined using an ABA radioimmunoassay

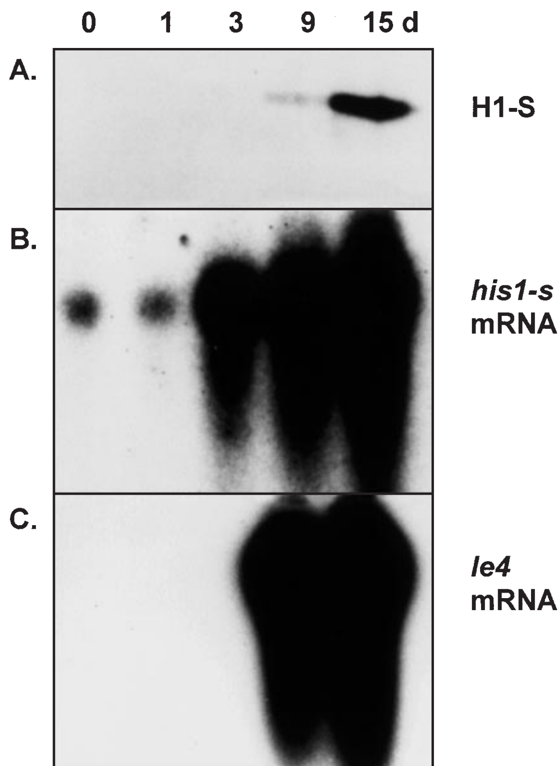

Fig. 3A–C. Protein and mRNA of leaves from whole tomato plants subjected to soil water deficit. Water was withheld from whole plants for 15 d. **A** Chromatin preparations were prepared from leaves at each time, subjected to SDS-PAGE according to Thomas and Kornberg (1975) and immunoblotted. The protein was detected with antiserum to H1-S from tomato. **B** RNA was extracted and separated by formaldehyde agarose gel electrophoresis, blotted and hybridized with the cDNA encoding H1-S. **C** The same RNA was hybridized with the cDNA representing the gene *le4*, a tomato dehydrin

expression of *his1-s* mRNA. After 3 d of water-deficit stress, the *his1-s* mRNA level was notably increased, reaching the maximum after 15 d (Fig. 3B). Similar to that observed for detached leaves, there was a lag period in the accumulation of H1-S protein. The H1-S protein could be detected only after 9 d, reaching the maximum accumulation after 15 d of water-deficit stress (Fig. 3A). The expression of another water-deficit and ABA-regulated gene, *le4*, was also measured in the plants subjected to soil water deficit; this mRNA could not be detected until day 9 (Fig. 3C).

The extent of stress does not alter H1-S protein accumulation. Leaves were subjected to different degrees of water loss and maintained in plastic bags for 24 h. The extent of water loss did not alter the accumulation of H1-S (Fig. 4). The same amount of protein was

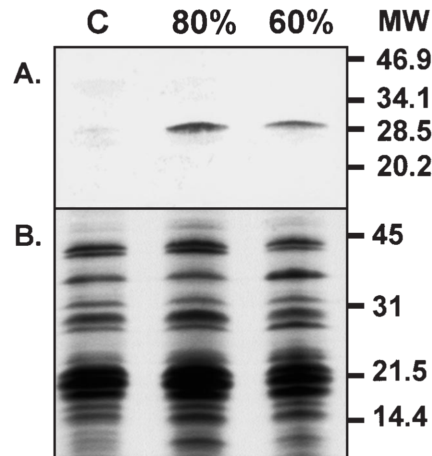

Fig. 4A,B. The effect of different degrees of water deficit on H1-S protein accumulation. Detached leaves were wilted to 80 or 60% of the original fresh weight and maintained in plastic bags for 24 h. Chromatin preparations were prepared from leaves, subjected to SDS-PAGE according to Thomas and Kornberg (1975) and immunoblotted. The protein was detected with antiserum to H1-S from tomato. **A** Immunoblot. **B** Coomasie blue-stained gel. The position of the molecular weight markers is indicated on the right side (*MW*)

detected after 80 and 60% loss of leaf weight. Correction for protein loading after scanning the Coomassie-stained acid-extracted protein profile indicated an even greater similarity.

H1-S has consensus nuclear localization signals. There are several recognized nuclear localization signals (NLS) in the deduced amino-acid sequence of H1-S. As shown in Fig. 5, a KKPR motif is located at amino acid 35, KKPK at 43, KKKP at 143, KKPR at 144, KKPKK at 170 and a Robbins and Dingwall nuclear consensus at position 157 (KKTEVPKKAKAT; Robbins et al. 1991; PSORT server: http://nibb.ac.jp). The presence of putative nuclear targets indicates that H1-S may be a nuclear protein but does not demonstrate the localization of the protein in vivo in response to cellular water deficit. The cellular localization of H1-S during water stress was investigated using three different approaches.

Cellular fractionation analyses. A biochemical approach, using cellular fractionation techniques, was used to identify the cellular localization of H1-S in tomato leaves subjected to water deficit. Histone H1 is an acid-soluble protein and can be extracted with H_2SO_4. The H_2SO_4-soluble proteins were extracted from total protein preparations, purified nuclei and chromatin preparations of non-stressed and wilted tomato leaves (Table 1). Using antiserum raised against H1-S tagged

MTAIGEVENPAVVQRPTEASKVKEQASATEKAVKE**KKPR**APKE**KKPK**SAK (50)
AVTHPPYFQMIKEALLSLNEKGGSSPYAVAKYMEDKHKDELPANFRKILG (100)
LQLKNSAAKGKLIKIKASYKLSEAGKKETTTKTSTKKLPKADS**KKKPR**ST (150)
RATSTAA<u>KKTEVPKKAKAT</u>P**KPKK**VGAKRTRKSTPAKAKQPKSIKSPAAK (200)
RAKKIAV (207)

Fig. 5. The amino acid sequence of H1-S with possible nuclear localization signals noted. H1-S contains four lysine-rich consensus sequences (*bold*) and a Robbins and Dingwall consensus (*underlined*; Robbins et al. 1991)

Table 1. Accumulation of H1-S in nuclear and chromatin preparations of detached leaves subjected to water-deficit stress or non-stress conditions. The leaves were fractionated to obtain a total protein preparation, a preparation of nuclei and a preparation of chromatin. Acid-soluble protein was isolated from these preparations. The protein was separated by SDS-PAGE, transferred to nitrocelluoluse and identified using antiserum to H1-S. The western blot and stained gel, which were run simultaneously, were scanned and the volume (recorded in arbitrary units) of the relevant bands was determined. The measure of H1-S was normalized for loading error by dividing the scanned value by the scanned value of the core histones and multiplied by 100

	Band volume (arbitrary units)			
	Non stress		Water deficit	
	H1-S volume	Normalized	H1-S volume	Normalized
Total	6,877	9.28	36,785	29.30
Nuclei	667	6.83	22,647	24.27
Chromatin	1,216	1.45	27,057	31.29

with His_6 and expressed in bacteria, immunoblot analyses indicated that H1-S is an acid-soluble protein of 31 kDa apparent molecular weight that is mainly associated with the chromatin. H1-S accumulated in the nuclei and in the chromatin of wilted leaves. The greater amount of protein recognized by the antibody in the total extract can be accounted for by differences in the amount of protein in each lane. When the amount of protein detected by the antibody was normalized for loading, it was concluded that the same amount of H1-S was observed in each of the preparations, indicating that the majority of H1-S was present in the nucleus (Table 1). In the absence of stress, however, the data may indicate that H1-S is not associated with the chromatin.

Immunolocalization. There is evidence that the accessibility of chromatin to an anti-H1 serum is dependent on the conformation of the chromatin (Takahashi and Tashiro 1979). Particularly, highly compacted chromatin inhibits the interaction of the antibody with the linker histones. To partially unfold the chromatin and make the protein antigenic sites available to the antibody, 4-μm cryosections of wilted and non-stressed leaves were treated with low salt (5 mM TBS). After the low-salt treatments, sections were fixed in formaldehyde, and incubated with antibody to H1-S, then with biotinylated secondary antibody and an extravidin-FITC conjugated fluorochrome. In order to identify the nuclei, the sections were stained with DAPI, which specifically binds DNA. Several nuclei identified with DAPI were detected by the H1-S antibody in the wilted leaves (data not shown). Without the low-salt treatment, the antibody reaction could not be detected. Non-stressed samples exhibited a very low signal, comparable to that of the pre-immune serum (data not shown).

Localization of an H1-S::GUS fusion in onion epidermis and transgenic tomato plants. Because of the difficulty in identifying the cellular localization of H1-S using immunofluorescence, a different approach was used to corroborate the biochemical data. Particle-gun bombardment was used to transiently express a construct containing H1-S fused with the reporter β-glucuronidase (GUS) in onion epidermis. Histochemical assays of onion epidermis transformed by particle-gun bombardment with the construct pBI221 (Fig. 6A) and pBI221-H1-S::GUS (Fig. 6B) were used to determine the sub-cellular localization of the fusion protein H1-S::GUS. When onion epidermal cells were bombarded with the construct pBI221, the blue dye was distributed throughout the cell (Fig. 6A). However, when GUS was fused to H1-S (pBI221-H1-S::GUS) and expressed in onion cells, GUS activity was primarily located in the nucleus (Fig. 6B).

To better investigate H1-S localization in tomato cells, the H1-S::GUS fusion was transferred from the vector pBI221 to the vector pBI121 and was used for *Agrobacterium*-mediated transformation of tomato leaf disks. Regenerated kanamycin-resistant tomato plants were analyzed for the cellular localization of H1-S by detecting GUS activity (Fig. 6D,F). Localization of the H1-S::GUS fusion protein was performed on cryosections (8 μm) of non-stressed and wilted detached leaves. The expression of the fusion protein was detected after 1 h of presentation of substrate. After the formation of blue precipitate, the sections were simultaneously stained with the DNA-specific fluorochrome DAPI (Fig. 6C,E). A blue precipitate accumulated in nuclei identified by DAPI in trichomes from tomato leaves and in leaf cross-sections (Fig. 6D,F) either under water-deficit stress or non-stress conditions, indicating that once the protein is synthesized it does not require water stress to accumulate in nuclei.

Discussion

H1 histones, together with the four core histones, are abundant basic proteins that compact DNA into the nucleosome structure. The nucleosome, in its role as the principal packaging unit of DNA, is the primary determinant of DNA accessibility and it regulates important DNA functions including recombination, replication, mitotic condensation and transcription (Kornberg and Lorch 1999). Linker histones are a family of isoprotein species encoded by multiple genes and the expression of these genes is dependent on the tissue, organ, and stage of development or environmental conditions. Several linker-histone subtypes have been isolated from maize (Ivanchenko et al. 1987), pea (Bers et al. 1992), *Arabidopsis* (Gantt and Lenvik 1991; Ascenzi and Gantt 1997), tomato species (Wei and O'Connell 1996; Bray et al. 1999), and tobacco (Szekeres et al. 1995). The expression of most of the linker histones isolated from plants is related to the cell cycle, since linker histones accumulate in proliferating tissues (Razafimahatratra et al. 1991; Jayawardene et al. 1994; Szekeres et al. 1995). The H1 histone identified in tomato by Jayawardene and Riggs (1994) is similar to

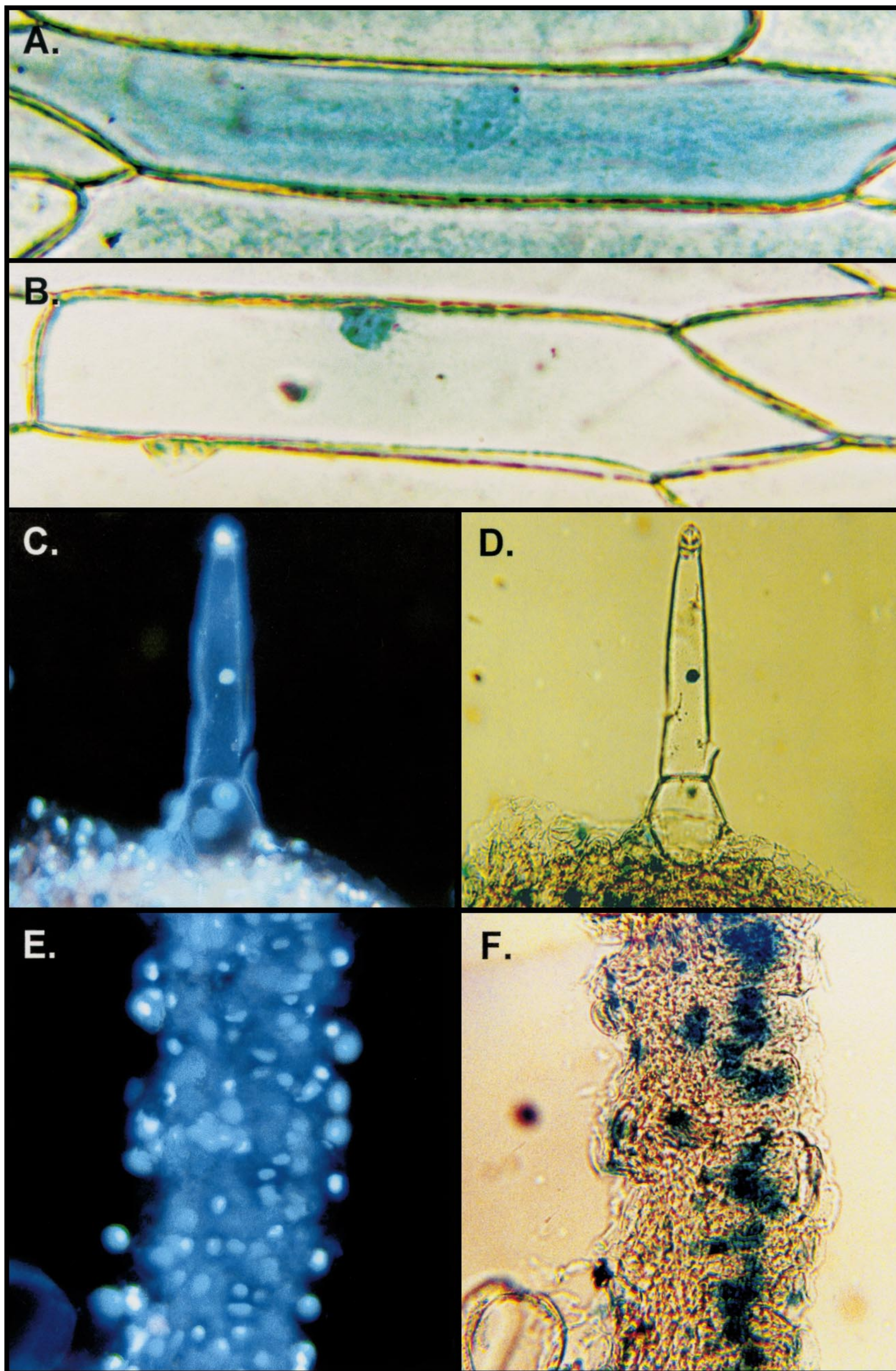

Fig. 6A–F. *β*-Glucuronidase activity in onion epidermis and in transgenic tomato expressing H1-S::GUS. **A, B** DNA encoding GUS (**A**) or H1-S::GUS with expression driven by the 35S promoter of CaMV (**B**) was delivered to onion epidermis for transient expression assays. Histochemical GUS assays were used to locate GUS activity within the cell. **C–F** Transgenic tomato plants were constructed containing H1-S::GUS driven by the 35S promoter. The position of the nuclei in the transgenic tomato line was identified using DAPI. **C** A trichome of tomato stained with DAPI. **D** Histochemical GUS activity of the same trichome showing GUS activity predominantly in the nuclei. **E** A leaf cryosection stained with DAPI. **F** *β*-Glucuronidase histochemical assay of the leaf cryosection

other known plant H1 histones and is highly expressed at the mRNA level in meristematic tissues.

The tomato H1 histone, *his1-s*, belongs to a different class of linker histones in plants. In fact, the amino acid sequence is divergent from that of the other H1 histones (Bray et al. 1999), and its expression is induced in differentiated tissues by ABA and water-deficit stress conditions (Bray et al. 1999). H1-S, together with H1-3 from *Arabidopsis* (Ascenzi and Gantt 1997) and His1 from *L. pennelii*, are unique examples of a plant linker histone induced by the environmental stress water deficit. The work reported here highlights the complex expression pattern of *his1-s*, shows the cellular localization of the *his1-s* gene product and strongly indicates that this is a linker-histone subtype.

Whole plants and detached leaves subjected to water deficit were used to analyze the expression of *his1-s* at the mRNA and protein levels. Interestingly, our data demonstrated that *his1-s* mRNA accumulates rapidly after water-deficit imposition and correlates with the accumulation of ABA (Figs. 1, 2, 3). However, H1-S protein accumulation occurs after a lag time (Figs. 1, 3) and it does not correlate with the level of ABA (Fig. 2). The expression of *his1-s* is complex since the expressed state of the gene, which is the functional protein product, may be regulated at multiple levels:

transcriptional, post-transcriptional, and translational. It was previously shown that *his1-s*, contrary to three other ABA-induced genes, is regulated in part at the post-transcriptional level (Bray et al. 1999). The accumulation of mRNA during water-deficit response may indicate gene induction, but additional regulatory mechanisms at the translational level may be required for gene product accumulation. At least two signal transduction pathways have been proposed for regulation of gene expression during the water-deficit response, an ABA-dependent and an ABA-independent signal transduction pathway (Shinozaki and Yamaguchi-Shinozaki 1997). We propose the presence of a second post-transcriptional regulatory step following mRNA accumulation that may be independent of ABA accumulation. It is hypothesized that the expression of *his1-s* follows the ABA-dependent pathway for mRNA accumulation, while protein accumulation requires other signals activated in a late response to water deficit following an ABA-independent pathway. A similar expression pattern has been shown for osmotin, a salt-stress-induced protein encoded by a gene that is transcriptionally induced in response to ABA, and translationally regulated by low water potential (LaRosa et al. 1992).

The particular pattern of expression shown here suggests a specialized role for H1-S that might be required only if the plant remains in a status of low water potential for a certain period of time. Plant responses to water deficit are complex, occurring within a few seconds, minutes or hours; responses may result from injury or promote adaptation to the new water status (Bray 1997). Two possible functions may be proposed for H1-S, a structural role in protecting the DNA from damage occurring during water deficit or a functional role in regulation of gene expression. H1 linker histone has been proposed to act as a general repressor of gene expression by changing chromatin conformation (Laybourn and Kadonaga 1991). Recently it has been shown that several linker-histone subtypes are involved in the activation or repression of specific genes (Bouvet et al. 1994; Shen and Gorovsky 1996). The data presented are not sufficient to address the question of the function of *his1-s* during water deficit but they open discussion. The cellular fractionation and H1-S::GUS analyses clearly showed that the *his1-s* gene product is a histone subtype localized in the nucleus and associated with the chromatin. However, no strong signals could be obtained using the immunolocalization approach. The failure of the immunolocalization techniques may indicate a technical problem, but they may also provide some clues to the localization of H1-S in the chromatin structure, which could be related to its function. These data may indicate that H1-S is localized in the interior of the chromatin 30-nm filament so that the epitopes are not available to the antibody. Furthermore, the protein could be distributed in specific regions, not throughout the chromatin. This interpretation of the immunolocalization data would exclude the role of H1-S in physical protection of DNA from damage associated with dehydration. If involved in the protection of DNA, it would be expected that H1-S would be more abundant, distributed throughout the chromatin, and easily accessible to the antibody. For example, dehydrin, a late-embryogenesis-abundant protein, is found throughout the nucleus and can be detected by immunolocalization techniques (reviewed by Close 1997). A more sustainable hypothesis may be the role of H1-S in the regulation of specific genes during water stress, since this function does not require the protein to be abundant and distributed throughout the chromatin.

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