A Novel Nucleus-Targeted Protein Is Expressed in Barley Leaves during Senescence and Pathogen Infection

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The barley (Hordeum vulgare) cDNA HvS40 represents a gene with enhanced mRNA level during leaf senescence. Biologic transformation of onion (Allium cepa) epidermal cell layers with a glucuronidase fusion protein construct provided evidence that the 15.4-kD protein encoded by HvS40 is localized to the nucleus. Expression of the gene is induced by jasmonate and salicylic acid; both are known to act as signaling compounds during senescence and defense toward pathogens. Transcript levels of HvS40 were observed to be particularly high in leaf sectors that undergo necrosis and chlorosis after infection with Pyrenophora teres. This pathogen-related expression is, in contrast, clearly reduced in transgenic barley plants expressing a stilbene synthase from grape (Vitis vinifera), whereas the mRNA level of a gene encoding the pathogen-related protein HvPR1 is unaffected. In situ hybridization with HvS40 antisense RNA revealed that during leaf senescence, the HvS40 transcript predominantly accumulates in the mesophyll. Taken together, the findings suggest a connection between the nuclear protein encoded by HvS40 and the degeneration of chloroplasts occurring during senescence and during infection of barley wild-type plants with P. teres.

Leaf senescence as the final stage of leaf development preceding cell death is characterized by degradation and remobilization of cellular material to growing leaves and developing seeds (for review on senescence, see Noodén, 1988; Matile, 1992). It is well established that leaf senescence is a highly ordered and complex process requiring the expression of specific genes (Buchanan-Wollaston, 1997; Nam, 1997; Quirino et al., 2000). Many genes whose transcripts are up-regulated during senescence (often referred to as senescence-associated genes [SAGs]) have been identified (Buchanan-Wollaston, 1997; Quirino et al., 2000). The functional diversity of the SAGs suggests that the leaf senescence program is highly regulated and involves various cellular activities, including degradation processes and maintenance of cell viability (Quirino et al., 2000).

Most SAGs are not only expressed during natural senescence (i.e. mediated by age), but respond to a variety of circumstances, including stress situations and hormones, which induce premature senescence (Parthier, 1990; Weaver et al., 1998). Two examples for this are jasmonate (JA) and ethylene, which both play a signaling role during plant defense and wounding (Titarenko et al., 1997; Pieterse and van Loon, 1999; Ryan, 2000). Recently, salicylic acid (SA), primarily known as a signaling compound during pathogen infection, has been shown to play a role during senescence (Morris et al., 2000). Thus, there is a considerable overlap in gene expression during leaf senescence and pathogen infection (Quirino et al., 2000).

Several members of different pathogenesis-related gene classes show enhanced transcript levels during senescence (Hanfrey et al., 1996; Quirino et al., 1999, 2000). Vice versa, some of the genes first described as SAGs were also found to be expressed in response to pathogens, e.g. the Brassica napus LSC 54 gene encoding a putative metallothionein (Butt et al., 1998) and the SAG12 gene encoding a Cys protease of Arabidopsis (Pontier et al., 1999). Because at least five of the defense-related genes of Arabidopsis are still expressed in senescing leaves under sterile conditions (Quirino et al., 1999), it seems likely that defense-related gene expression is an integral part of the senescence program rather than a direct response to pathogen infection (Quirino et al., 2000). Senescence and pathogen infections have certain features in common, e.g. programmed cell death (Pennell and Lamb, 1997), chlorosis, and an enhanced activity of enzymes involved in cellular turnover and in scavenging of potentially damaging metabolites (Greenberg and Ausubel, 1993).
In many studies, dark incubation has been employed to induce senescence. Among the genes expressed under this condition (Kleber-Janke and Krupinska, 1997; Park et al., 1998), some seem to be primarily stress related and are not as strongly expressed during natural senescence (Becker and Apel, 1993; Weaver et al., 1998). So far, only a few genes have been identified that primarily are expressed during natural senescence and do not respond more strongly to other factors. One of these genes is SAG12 of Arabidopsis (Weaver et al., 1998). In the case of barley (Hordeum vulgare), expression of the HvS40 gene, although also induced by darkness (Becker and Apel, 1993; Kleber-Janke and Krupinska, 1997), has served as a reliable molecular marker of age-mediated leaf senescence. The expression of HvS40 inversely correlates with the decline in photosynthetic efficiency (Humbeck et al., 1996).

In this paper, we show that expression of the HvS40 gene is induced by the signaling compounds JA and SA, respectively. In addition, we investigated expression of the gene during infection of barley leaves with fungal pathogens. Sequence analyses revealed that the gene product possesses two motifs that resemble nuclear localization signals (NLSs). Expression of a fusion of HvS40 with the uidA gene encoding the glucuronidase (GUS) protein confirmed the nuclear localization of the protein. To our knowledge, HvS40 is the first senescence-related gene whose gene product is transferred to the nucleus.

RESULTS

cDNA Isolation and Analysis of the Deduced Amino Acid Sequence

A partial cDNA (310-bp length) was isolated from a cDNA library representing gene expression 2 d after the onset of senescence of flag leaves under field conditions (Humbeck et al., 1996). The sequence of this 310-bp cDNA turned out to be identical with the sequence of the HvS40 cDNA (W. Becker, personal communication) from a cDNA library representing genes expressed in dark-treated detached primary foliage leaves of barley (Becker and Apel, 1993). To isolate the complete cDNA, the corresponding fragment was used for rescreening a cDNA library prepared from senescing flag leaves (Kleber-Janke and Krupinska, 1997). The largest cDNA clone contains an insert sequence (608-bp length) with six nucleotides 5' to the first ATG (accession no. AJ310379). The sequence surrounding the first ATG has high relatedness to the transcription start sites of other plant mRNAs (Cavener and Ray, 1991). The coding sequence is terminated by a stop codon at position 421 and, hence, encodes a putative protein of 138 amino acids. Two other possible reading frames would encode putative peptides of 99 and 47 amino acids starting with ATG codons at positions 239 and 423, respectively. Codon usage analyses suggest, however, that the largest reading frame starting with an ATG at position 7 is used.

The deduced HvS40 protein is rich in basic amino acids (17.3%), mainly Arg (13%) and Lys (4.4%). Database analyses showed significant homologies to the amino acid sequences of several expressed sequence tags (ESTs) and two putative proteins deduced from the genomic sequences of Arabidopsis and rice (Oryza sativa). Among the EST sequences from barley are several identical with the DNA sequence of HvS40 and a second group, represented by the sequence named Hordeum 2, with significant homologies appearing only at the amino acid level (Fig. 1). Among the EST sequences from Triticum aestivum, several are related to HvS40 and others are related to Hordeum 2. At least four predicted protein sequences from rice with homologies to the HvS40 amino acid sequence are available, of which two are included in Figure 1. Overall, up to 16 hypothetical proteins with high homology to the carboxy-terminal part of the HvS40 protein could be identified in the genome of Arabidopsis, of which two are depicted in Figure 1. The hypothetical proteins from Arabidopsis and one from rice have been pooled as protein family Pfam-B 2580 in the Pfam database (Bateman et al., 2002). However, no function has yet been assigned to any of these putative proteins.

As predicted by the PSORT program, the protein sequence of HvS40 contains two putative NLSs (Fig. 1). The first motif consisting of the four basic amino acids RRKR belongs to the class of SV40 nuclear target motifs and the second motif belongs to the bipartite class (Raikhel, 1992) and comprises the amino acids KRNVTPHVLERR. These NLS are not preserved in the other sequences depicted in Figure 1. However, at least one alternative NLS is present in the second barley protein.

The conserved part of the sequence starts around position +70 (Fig. 1) with the sequence motif SAPV. This motif is present in almost all homologous sequences. The following parts of the sequences are highly variable in length and in all cases contain a high number of the acidic amino acids Asn and Glu and are terminated by highly conserved Pro and His residues. The putative HvS40 protein contains the first NLS in this region. The last 35 to 40 amino acids are highly conserved among all sequences. The highly conserved C-terminal region contains three potential phosphorylation sites (Fig. 1), which are present in all sequences presented in Figure 1 and are present in almost all sequences containing this motif. At the C-terminal end, a conserved TGF box is followed by two or three amino acids.

Estimation of Gene Copy Number

Southern-blot analyses with barley genomic DNA restricted by XbaI and XbaI/XhoI, respectively, suggest that the barley cDNA HvS40 represents a single-copy gene (Fig. 2).
Subcellular Localization of an HvS40-GUS Fusion Protein

The 3’ end of the complete HvS40 cDNA was ligated in frame to the uidA gene encoding the GUS protein. The construct was then used for biolistic transformation of onion (Allium cepa) epidermal cell layers. The onion transformation system is well suited to demonstrate the location of heterologous proteins, e.g. the maize (Zea mays) opaque-2 protein (Varagona et al., 1992). After incubation of epidermal cell layers in 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc) solution, GUS staining was observed predominantly in nuclei (Fig. 3). In the case of the uidA control construct without the HvS40 gene, staining was obtained in the cytoplasm only.

HvS40 Expression in Response to JA and SA

To gain insight into the regulation of HvS40 gene expression, mRNA levels in barley leaves were analyzed after incubation with JA and SA, which are believed to have signaling functions during leaf senescence (Parthier, 1990; Morris et al., 2000). The levels of both compounds in leaf tissue are known to increase during senescence in Arabidopsis (Morris et al., 2000; He et al., 2002). JA levels were also shown to increase during senescence in barley leaves (C. Wasternack, personal communication). Segments of barley primary foliage leaves were floated for up to 120 h on either 100 μM JA or 1 mM SA solubilized in tap water, respectively. Control segments were floated on tap water. As shown in Figure 4, both compounds induced expression of the HvS40 gene. Although expression in response to JA was detected already after 16 h of incubation, expression in response to SA was detected not before 40 h. Similar results were obtained when instead of SA, acetyl-SA was used (data not shown).

Expression of the HvS40 Gene in Response to Infection with Fungal Pathogens

Responsiveness to JA and SA suggests that the HvS40 gene may encode a pathogen-related protein.
JA is known as a key signaling compound during wounding (Creelman and Mullet, 1997; Ryan, 2000) and also may play a role during fungal infections (Titarenko et al., 1997). SA is involved in various plant-pathogen interactions (Durner et al., 1997). It plays a key role in activation of systemic-acquired resistance associated with the expression of defense genes such as pathogenesis related genes (Hammond-Kosack and Jones, 1996) as well as in the local induction of defense genes (Delaney et al., 1994). To investigate whether expression of HvS40 is induced during plant-pathogen interactions, barley leaves were infected with the perthotrophic fungus Pyrenophora teres (Keon and Hargreaves, 1983; Ruiz-Roldán et al., 2001) by local application of a spore suspension to sectors of leaves attached to the plants. After incubation for 5 d using small plastic bags to cover the infection sites, RNA was isolated from infected leaf sectors, from uninfected parts of the same leaf, from another leaf of the same plant, and from corresponding leaf material of control plants, which had been treated likewise, but without spores. Northern-bolt analysis showed that the HvS40 gene was strongly expressed exclusively in the infected leaf sectors, which had developed necrotic lesions surrounded by chlorotic zones after 5 d of incubation (Fig. 6A). In comparison, expression of the HvPR1a gene is not restricted to the infection sites, but is clearly detectable also in the uninfected tissue of the

![Figure 3](image-url)  
Subcellular localization of the HvS40-GUS fusion protein in epidermal onion cells. A control construct with the uidA gene (A and B) as well as the HvS40-GUS fusion construct (C and D) under the control of the 35S cauliflower mosaic virus (CaMV) promoter were introduced into the cells by biolistic transformation. Staining in X-gluc solution was performed overnight before microscopic analysis. Magnifications are 10× (A and C) and 40× (B and D).

![Figure 4](image-url)  
Northern-blot analyses of the HvS40 transcript level in barley primary foliage leaf segments in response to methyl JA and SA. A, Leaf segments from 6-d-old barley seedlings were floated either on water or on solutions of methyl JA and SA, respectively. Incubation was performed for up to 120 h in continuous light. B, To confirm equal loading of RNA, the filter was stained with methylene blue.

![Figure 5](image-url)  
Chlorophyll content (A) and PSII efficiency measured by fluorescence (B) of barley leaf segments after incubation for up to 120 h on solutions of 1 mM SA (■), 100 μM methyl JA (▲), or on tap water (●).
same leaf and weakly in other leaves of the plants used for infection (Fig. 6A). Infection of barley cv Sultan 5 with the biotroph Erysiphe graminis, which is an example for a resistant interaction accompanied by hypersensitive cell death in attacked epidermal cells and adjacent mesophyll cells (Hückelhoven et al., 2000), resulted in a very weak expression of the HvS40 preceded by expression of the HvPR1a gene (data not shown).

To investigate whether expression of the HvS40 gene is related to the severity of necrotic lesion formation by P. teres or is part of the plant’s defense against the infection, expression was analyzed in a transgenic barley line (53/1) expressing the stilbene synthase gene of grape (Vitis vinifera; Leckband and Lörz, 1998). As demonstrated before, this transgenic line had a higher resistance toward the pathogenic fungus Botrytis cinerea. After infection with B. cinerea, leaves stayed greener and developed smaller necroses at the sites of infections, which could probably be traced back to a hypersensitive reaction (Leckband and Lörz, 1998). When inoculated with spores from P. teres, leaves of this line stayed almost green in comparison with those of the wild type and developed fewer necroses (Fig. 6C). As shown in Figure 6B, infected sectors of leaves of the line 53/1 contained a significantly lower level of the HvS40 compared with the corresponding sectors of leaves from wild-type plants. In contrast to HvS40 expression, HvPR1a expression showed almost no difference between wild-type plants and transgenic plants (Fig. 6, A and B). Taken together, these results suggest that HvS40 expression in response to pathogens occurs exclusively at the sites of necrotic lesions surrounded by chlorotic tissue.

**Predominance of HvS40 Transcripts in Mesophyll Cells**

To investigate the spatial expression of the HvS40 gene in barley leaves, transverse sections from primary foliage leaves were used for in situ hybridizations. Plants were grown for 9 d in a daily light/dark cycle. Thereafter, some of the plants were additionally transferred for 3 d into darkness. With HvS40 antisense probes, signals were detected in the mesophyll of senescing leaves with the exception of the inner cells of the vascular tissue (Fig. 7). Background signals in the epidermal cells were observed both in leaves before transfer into darkness and in leaves induced to senesce by darkness (Fig. 7) as well as with the sections from senescent leaves after hybridization with an HvS40 sense probe (Fig. 7).

**DISCUSSION**

The protein encoded by the HvS40 gene of barley is the first SAG gene product shown to be targeted to the nucleus as indicated by sequence analysis and as confirmed by heterologous transformation of onion epidermal cells. The HvS40 protein is a rather small protein with a predicted molecular mass of 15.4 kD. In principle, proteins smaller than 40 kD can diffuse through the nuclear pores. However, there are many examples of small proteins, which nevertheless possess an NLS, e.g. the 20-kD histones. There is no unambiguous consensus sequence for an NLS. Typically, they contain Arg and Lys residues (Raikhel, 1992). The nuclear HvS40 protein belongs to the group of nuclear proteins that possess two putative NLSs, one belonging to the SV40 class, the other to the class of bipartite NLSs. In some cases, multiple NLSs are necessary for nuclear targeting (Raikhel, 1992). Because the GUS-HvS40 fusion construct contains both NLSs, we cannot predict whether one of the two NLSs would be sufficient for nuclear localization. In the case of the maize transcription factor opaque 2, the bipartite NLS has an additional function in DNA binding. Database analyses of the bipartite NLS of the S40 protein, however, did not reveal DNA-binding properties of this region. Because the

![Figure 6](https://www.plantphysiol.org/content/130/3/1176/F6.large.jpg)

**Figure 6.** Accumulation of HvS40-specific transcripts in response to infection with P. teres. Northern-blot analysis with RNA derived from different leaf samples of barley wild-type plants (A) and transgenic line 53/1 overexpressing a stilbene synthase gene (B) 5 d after infection with spores from P. teres: 1, leaf segments with infection sites; 2, sectors of the same leaf without infection sites; 3, another leaf of the infected plant; and 4, a leaf of a control plant treated likewise as the infected leaf but without spores. The RNA filter was first hybridized with the HvS40-specific probe and then rehybridized with a probe specific for the HvPR1a protein. C, Photograph of an infected leaf from a wild type (left) and the transgenic plant 53/1 (right) 5 d after infection.
NLSs are not conserved among the homologous sequences, it can be proposed that the function of the highly conserved C-terminal domain is not related to the nuclear localization of the proteins.

Southern-blot analysis with total barley DNA suggests that HvS40 is a single-copy gene. By homology search within the barley EST collection, a second gene with significant homology only at the amino acid level was detected (Hordeum 2 in Fig. 1B). Both barley proteins have high homology to putative protein sequences from Arabidopsis and rice, which had been grouped into the Pfam-B2580 family (Bateman et al., 2002). Although the predicted proteins from most genes of the Pfam-B2580 family have about the same size as the HvS40 protein, these Arabidopsis and rice genes are not significantly homologous to the HvS40 gene at the nucleotide level. Therefore, it is unlikely that the great number of proteins containing the Pfam-B2580 motif have arisen from simple duplication events. More likely, they might be the result of a modular shuffling of protein motifs to create new functions by reusing successfully invented motifs. One can further speculate that this highly conserved motif is essential for the function of these proteins regardless of their subcellular localization.

Expression of the HvS40 gene is induced by JA and SA, respectively. Although expression in response to JA occurs rather fast and is clearly detectable after 16 h, expression in response to SA requires more than 40 h of incubation. Because SA is a key signaling compound in plant pathogen interactions (Dürner et al., 1997) and JA may play a role in wounding that also occurs during infections by fungal pathogens (Titarenko et al., 1997), it is likely that the HvS40 gene belongs to the subset of senescence-related genes that are also expressed in response to pathogens (Quirino et al., 1999, 2000).

To investigate the HvS40 gene expression in response to pathogens, barley leaves were infected with two fungal pathogens differing in their infection strategies. Infection of barley leaves by P. teres is an example for a susceptible interaction, whereby cell death by necrosis occurs later than in the resistant response associated with a rapid cell death (hypersensitive response; Greenberg and Ausubel, 1993). P. teres penetrates an epidermal cell and develops intracellular infection vesicles that may act like haustoria. After breaching the epidermis, the fungus grows exclusively in the apoplast of the mesophyll tissue (Keon and Hargreaves, 1983). By its way of infection, it is likely that P. teres induces a wound response associated with the production of JA and ethylene at an early stage of infection (Titarenko et al., 1997; León et al., 2001). At a later stage, when hyphae penetrate the mesophyll, toxic peptides are secreted, poisoning the surrounding cells. Five days after infection, HvS40 gene expression was clearly detectable at the sites of infection showing necrotic lesions surrounded by chlorotic zones. In contrast, the gene encoding HvPR1a was also expressed systemically in other tissues. In comparison with barley wild-type plants, leaves of the transgenic barley line 53/1 overexpressing a stilbene synthase (Leckband and Lörrz, 1998) stayed almost green, developed fewer necroses, and had a much lower level of HvS40 mRNA at the sites of infection. In contrast to HvS40 gene expression, the expression of HvPR1a was barely different between wild-type and transgenic plants. It is possi-
ble that a higher level of antioxidants in leaves of the transgenic line may suppress the induction of the HvS40 gene.

Resistant interaction of barley cv Sultan 5 with the biotroph E. graminis is characterized by rapid cell death in a hypersensitive reaction (Hückelhoven and Kogel, 1998; Hückelhoven et al., 2000). In this case, a weak and rather late expression of the HvS40 gene was observed (data not shown).

It is striking that expression of the HvS40 gene in barley leaves infected by P. teres is restricted to the areas of infection showing necrotic lesions surrounded by chlorotic zones. Similarly, the SAG of Arabidopsis (SAG12) is expressed only in the chlorotic tissue surrounding the sites of hypersensitive reactions during infection of tobacco (Nicotiana tabacum) with tobacco mosaic virus (Pontier et al., 1999). As mentioned before, during infection with the biotroph E. graminis, a weak expression of the HvS40 gene was detected (data not shown). This expression occurs, however, rather late and is likely to be related to the attack of the mesophyll and the formation of superoxide in the chloroplasts (Hückelhoven and Kogel, 1998). Hence, expression of HvS40 during pathogen interactions resembles expression of LSC54 from B. napus. LSC54 is expressed during an incompatible interaction in cells neighboring those cells that undergo a hypersensitive response, and is expressed rather late during compatible interactions, when the tissue turns yellow (Butt et al., 1998).

The results show that HvS40 expression is not related to fast cell death, but rather to senescence-like processes occurring in the tissue surrounding the infection sites of P. teres and in the mesophyll of leaves adjacent to the epidermal cells infected by E. graminis. Because expression of the HvPR1a and HvS40 genes differ considerably under the various conditions of interactions with fungal pathogens, HvS40 gene expression seems not to be related to the defense against pathogens.

The results on HvS40 gene expression in response to the different pathogens are in accordance with an expression of the gene restricted to the mesophyll cells as shown by in situ hybridization with HvS40 antisense RNA using transverse sections from leaves induced to senescence by darkness.

In this paper, we showed that the barley HvS40 gene responds to both signaling compounds, SA and JA. JA is a stronger inducer than SA. It is possible that during infection with P. teres, JA is produced as a consequence of an accumulation of reactive oxygen species (ROS), e.g., superoxide. Superoxide may be formed via ferredoxin/NADPH oxoreductase as a consequence of impairment of the photosynthetic apparatus.

In contrast to the SAG12 gene of Arabidopsis, which seems to be induced only by treatments that lead to a visible chlorosis (Weaver et al., 1998), expression of the HvS40 gene as well as of the LSC54 gene of B. napus (Morris et al., 2000) is also induced by SA, which does not lead to chloroplast breakdown. Hence, besides a JA/ROS-dependent signaling pathway a second signaling pathway for induction of the HvS40 gene has to be envisaged. In Arabidopsis mutants defective in the SA signaling pathway, expression of some SAGs clearly depends on the presence of SA (Morris et al., 2000). One model is that application of SA leads to an increase in hydrogen peroxide by inhibiting catalase activity (Durner et al., 1997). Further studies are in progress to clarify whether differences in the subcellular localization of ROS produced after application of JA and SA, respectively, may account for differences in the kinetics of HvS40 gene expression.

Taken together, HvS40 gene expression is primarily related to the breakdown of the photosynthetic performance occurring during leaf senescence, as a consequence of JA treatment and of pathogen infections involving the mesophyll and leading to chlorosis. In this regard, pathogen infection simply seems to be a stimulus inducing senescence-like processes. Under all these conditions, ROS could act as a trigger for HvS40 gene expression. This hypothesis is in accordance with the lower level of expression in transgenic plants overexpressing the stilbene synthase gene and having a higher content of the phenolic antioxidant resveratrol (Wu et al., 2001).

MATERIALS AND METHODS

Plant Material and Bioassays

Barley (Hordeum vulgare L. cv Carina) seedlings were grown for 6 d at 21°C on moist vermiculite under constant illumination with white light of 130 μE m−2 s−1. For incubation with signaling compounds, leaf segments were excised from primary foliage leaves and were floated either on tap water or on tap water containing 1 mM SA or 100 μM methyl JA. The pH of the solution containing SA was 7.7. All incubations were done under continuous illumination. After incubation, 0.5 cm was removed from the cut edges and the segments were immediately frozen in liquid nitrogen.

For infection with Pyrenopeziza teres, leaf sectors of barley cv Igri plants (wild type), as well as the transgenic line S3/1 (Leckband and Lötz, 1998), were cultivated in a greenhouse and inoculated with 50 to 100 spores suspended in 20 μL of 0.01% (w/v) Tween 20. The infected area of these leaves was then covered by a small plastic bag. Five days after inoculation, the infected leaf area was chlorotic and showed necrotic lesions at the sites of infection. For RNA analyses, the infected leaf areas from several plants were excised and pooled. In addition, tissue without necroses of the same leaves and other leaves of the same infected plants were collected. Corresponding samples were taken from control plants treated likewise, but without spores. The leaf material was immediately frozen in liquid nitrogen.

cDNA Library Subtraction and Screening

cDNA libraries representing gene expression in flag leaves from field-grown barley plants (Humbeck et al., 1996) were prepared using the phage λZAPII as described previously (Kleber-Janke and Krupinska, 1997). Subtractive hybridization of a library representing gene expression 2 d before and 2 d after the onset of senescence was performed by the method of Schweinfest et al. (1990) using single-stranded phagemid DNA obtained by in vivo excision. For in vivo excision, the Exassist interference-resistant helper phage with the SOLR strain (Hay and Short, 1992) was used. The partial cDNA (310-bp length) was used for rescreening the λZAPII library to isolate the complete cDNA. Plaque lifts were made onto Hybond N+ mem-
The Senescence-Associated HvS40 Protein

Analysis of Chlorophyll Content and in Vivo Fluorescence

Chlorophylls were extracted with hot methanol and measured spectrophotometrically. Chlorophyll concentrations were calculated using the formula of Lichtenthaler (1987). Chlorophyll fluorescence was measured at room temperature using a pulse-amplitude modulated fluorometer (Walz, Effeltrich, Germany) as described by Humbeck et al. (1996). The measurements were performed at the mid-position of leaf segments. Mean values of the ratio variable fluorescence/maximum fluorescence ($F_v/F_m$) were based on at least five independent measurements.

In Situ RNA Hybridization

In situ hybridizations were performed with the method of Morrison and Leech (1994) using transverse sections from barley leaves fixed in 3% (w/v) paraformaldehyde, 50% (v/v) ethanol, and 5% (v/v) acetic acid, and embedded in polyethylene glycol 1500. Fifteen-micrometer sections were used for hybridizations with DIG-labeled sense or antisense RNA produced from the partial HvS40 cDNA by in vitro transcription reaction. The labeling reaction was performed with 1 μg of linearized template DNA, DIG-11-UTP, and T3- or T7-RNA polymerases according to the manufacturer’s instructions (Roche Diagnostics). The hybridized probe was detected using an anti-DIG-alkaline phosphatase conjugate and overnight color development with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as described by Marrion and Leech (1994).

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LITERATURE CITED


