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RESEARCH PAPER

High-throughput virus-induced gene-silencing approach to assess the functional relevance of a moisture stress-induced cDNA homologous to *lea4*

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Abstract

The abiotic stress-responsive cDNA database and their expression profiles suggest that stress genes are many and diverse. However, characterization and validation of their functional significance has been a constraint to assessing their role in imparting tolerance. Virus-induced gene silencing (VIGS) is a potential option for assessing the functional significance of stress genes. Here the effectiveness of VIGS to silence the expression of an ABA-responsive *lea4* (late embryogenic abundant) gene involved in stress tolerance is documented. In the present study, low moisture-stress protocols were developed in such a way that the plants experienced the desired stress level when silencing the target stress gene using VIGS was at a maximum. The functional relevance of a groundnut (*Arachis hypogaea*) subtracted-stress cDNA clone putative *lea4* was examined by VIGS in tomato. A 400 bp fragment of *lea4* was cloned into tobacco rattle virus-based VIGS vector to trigger post-transcriptional gene silencing by *Agrobacterium*-mediated inoculation in tomato plants. In silenced plants only *lea4* transcripts showed a substantial decline, whereas the expression of other known stress-responsive genes such as *apx* (ascorbate peroxidase) and *elip* (early light-induced protein) were unaltered. Under moderate moisture stress, the silenced plants showed enhanced susceptibility as measured by cell viability, superoxide radical activity, and cell osmotic adjustment. This approach illustrates the potential benefits of VIGS in identifying functional relevance of low moisture stress-responsive genes. It is also demonstrated that heterologous probes with a fairly high degree of homology to the native genes can be used to study the functional relevance of stress-responsive genes using VIGS.

Key words: LEA4, low moisture stress-induced genes, plant stress functional genomics, VIGS.

Introduction

A comprehensive understanding of the network of genes, proteins, and small molecules that underline plant stress responses requires identification and characterization of the diverse genes that respond to stress. A stress-specific transcriptome analysis might provide vital clues about the genes involved in bringing about the altered metabolism for adaptation. Several stress-specific functional and upstream genes were cloned, and a number of EST (expressed sequence tag) databases representing stress-induced transcripts have been developed in a few plant species (Seki *et al*., 2001*a*, *b*). Using a microarray approach, the expression pattern of a number of stress-inducible genes have been identified for diverse stresses (Seki *et al*., 2002*a*).

Genomic approaches have suggested that stress adaptation is a complex feature involving different genes associated with signal transduction and diverse functional genes that are involved in altering the metabolism (Bohnert *et al*., 2001; Krebs *et al*., 2002; Vinocur and Altman, 2005). Recently, from a cDNA microarray containing 7000 independent *Arabidopsis* full-length cDNA clones, several stress inducible genes including 299 drought-, 54 cold-, 213 salinity-, and 245 ABA-inducible genes were identified (Seki *et al*., 2002*a*, *b*; Shinozaki *et al*., 2003). Similarly, many stress-inducible genes have been identified using a gene strip array containing oligonucleotides for about 8000 independent *Arabidopsis* genes (Zhu *et al*., 2001). However, characterization of these stress genes is based on sequence homology, and the functional relevance of only a few of these genes has been examined. Therefore a critical step in exploiting these genomic resources depends on the

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development of novel tools and approaches for the functional analysis of the identified genes/ESTs.

The functional relevance of stress-responsive genes is being elucidated either by overexpression (Mahalakshmi *et al*., 2006) or down-regulation studies. In this regard, several down-regulation approaches like transposon or T-DNA insertional mutagenesis are being extensively used. Recently, virus-induced gene silencing (VIGS) emerged as a functional genomic tool for down-regulating the expression of target plant genes (Holzberg *et al*., 2002; Liu *et al*., 2002*a*, *b*; Dinesh-Kumar *et al*., 2003; Burch-Smith *et al*., 2004). Compared with the stable transformation of plants, the use of virus vectors has the advantage, since the time involved in cloning the gene of interest and analysis of the phenotype is relatively short. In addition, VIGS allows analysis of genes which otherwise show a lethal phenotype in T-DNA-tagged knockdown mutants or when silenced using methods which employ stable transformation (Baulcombe, 1999).

VIGS vectors are developed by modifying the viral genome to include host sequences to the corresponding host RNAs. These vectors with the gene of interest are introduced to the host plant and become the target for silencing, and the symptoms in the infected plant resemble the phenotype of a null or a reduced-function mutant (Kumagai *et al*., 1995; Ruiz *et al*., 1998). This approach of suppressing the host gene expression is useful in assigning gene function (Baulcombe, 1999; Waterhouse *et al*., 2001). Based on this concept, the functional relevance of diverse genes has been validated using the VIGS approach (Atkinson *et al*., 1998; Kjemtrup *et al*., 1998; Liu *et al*., 2002*a*).

The efficiency of silencing by VIGS has been studied extensively using viral vectors with the *pds* gene, which encodes the phytoene desaturase enzyme involved in carotenoid biosynthesis. Silencing the endogenous *pds* gene leads to a typical photo-bleaching phenotype. Apart from *pds*, other genes which cause visible symptoms upon silencing, namely *rbcs* (Liu *et al*., 2002*a*), sub-unit of magnesium chelatase complex (Kjemtrup *et al*., 1998; Peele *et al*., 2001), proliferating cell nuclear antigen sulphur (su) (Peele *et al*., 2001), and Chlorata 42 (Turnage *et al*., 2002) have also been used to study the efficiency of silencing in different plant species (Ruiz *et al*., 1998; Liu *et al*., 2002*a*, Turnage *et al*., 2002). By VIGS, functional characterization of a number of diverse genes involved in metabolic processes (cellulose synthase A) (Burton *et al*., 2000), responses against pathogens (tobacco Rar1, EDS1, and NPR1/NIM1) and genes associated with ethylene responses (CTR1) (Liu *et al*., 2002*a*) have been demonstrated.

In addition to the tobacco rattle virus (TRV)-based vector which is the most widely used VIGS vector for silencing studies in *Nicotiana benthamiana* and tomato (Liu *et al*., 2002*a*), efforts have been made in recent years to develop VIGS vectors for different hosts. For example, vectors have been developed for barley using barley stripe mosaic virus (Holzberg *et al*., 2002) and for *Arabidopsis thaliana* using cabbage leaf curl virus (Turnage *et al*., 2002).

Since the host species-specific VIGS vectors are limiting, the relevance of specific genes has been studied using heterologous probes in model systems like *Nicotiana benthamiana* and tomato using TRV-based VIGS vectors (Benedito *et al*., 2004). For optimal results leading to specific, effective, and reliable gene silencing in a heterologous system, establishment of the level of sequence homology is a prerequisite. With 74% nucleotide identity, the *pds* gene from *Nicotiana benthamiana* failed to provoke the desired level of silencing in barley. It is generally assumed that 85% nucleotide identity is the lower limit for triggering the silencing mechanisms (Holzberg *et al*., 2002). Therefore a fairly high degree of homology with an endogenous gene is necessary for down-regulation of the gene of interest. In addition, the extent of spread of viral transcripts and effective duration of silencing of the endogenous gene are important parameters that decide the effectiveness of VIGS in any plant system (Burch-Smith *et al*., 2004).

Although functional significance of several biotic and developmental specific genes have been validated using VIGS, information on VIGS protocols for the characterization of abiotic stress-responsive genes is lacking. As abiotic stress-responsive cDNAs/ESTs are many, a high-throughput knockout approach like VIGS will provide useful information for their functional characterization.

In this study, a protocol was developed to analyse the functional relevance of the abiotic stress gene *lea4* from groundnut by VIGS. Experimental evidence is provided to show that heterologous probes can be efficiently used to silence the endogenous genes. Silencing of *lea4*, a known stress-responsive gene involved in desiccation-stress tolerance, resulted in greater susceptibility of tomato plants to water-deficit stress, demonstrating for first time the use of this approach to assess the functional relevance of abiotic stress genes.

Materials and methods

Plant material and growth conditions

Tomato (cv. Arka vikas) seeds were obtained from the Indian Institute of Horticultural Research, Bangalore. Plants were grown in plastic pots holding 5 kg of potting mixture consisting of red soil:sand:vermicompost in the ratio of 3:1:1 by vol. (22% water-holding capacity). The recommended fertilizers including micro-, secondary, and macro-nutrients were provided. Plants were maintained in a temperature-controlled growth room with a temperature range of 22–25 °C, a relative humidity of 60%, and a 12 h photoperiod with light intensity ranging from 300 to 400 $\mu E\ m^{-2}\ s^{-1}$. Prophylactic measures were taken to maintain the plants disease/pest free.

TRV vectors and Agro infection (plant transformation)

The details of the TRV vector constructs namely pTRV RNA1 and pTRV RNA2 are described by Liu *et al*. (2002*a*, *b*). These vectors and their *pds* gene derivatives were kindly provided by SP Dinesh-Kumar, Yale University, USA. pTRV2 groundnut *pds* was provided by Kiran Mysore of The Noble Foundation, USA.

The pTRV2 derivatives: A subtractive-stress cDNA library was constructed from groundnut plants subjected to gradual low-moisture stress (Gopalakrishna, 2001). A partial cDNA clone, gsi87 (groundnut stress induced, accession number DQ256366) was selected from this library for VIGS studies. This clone has 97% nucleotide identity with gsi22 (NCBI accession number AF479307), another clone from the stress library coding for putative *lea4*. The gsi87 (400 bp) was sub-cloned into a TRV RNA2-based binary vector pYL156 under a 2× CaMV35S promoter (hereafter referred as pTRV2 *lea4*). Further, pTRV2 *nbpds* with a 360 bp gene fragment of phytoene desaturase (*pds*) from *Nicotiana benthamiana* (NCBI accession number AJ571700), pTRV2 *tompds* with a 400 bp fragment of *pds* from tomato (NCBI accession number M88683), and pTRV2 *gnpds* with a 341 bp gene fragment of *pds* from groundnut (NCBI accession number DQ117940) were used for the initial studies. TRV RNA1 vector (pTRV1) or pTRV2 derivatives were mobilized into *Agrobacterium* strain GV3101 by the calcium chloride method of transformation (Sambrook and Russell, 2001).

Agrobacterium tumefaciens cells carrying pTRV1 and pTRV2 or pTRV2 derivatives were cultured initially in 5 ml liquid broth media containing 100 μM MES buffer (pH 5.5) and then inoculated to a large culture of 50 ml and grown overnight at 28 °C (Liu *et al*., 2002*b*). *Agrobacterium* cells were harvested and resuspended in infiltration media (5 mM $MgCl_2$, 1 mM MES buffer, 200 μM acetosyringone; final pH 5.5), adjusted to an absorbance (OD 600) of 1.5, and incubated at room temperature for 3 h. Further, the respective *Agrobacterium* cultures were resuspended in 100 μM MES buffer and infiltrated to tomato using a needle-less 2 ml syringe (Liu *et al*., 2002*b*). Infiltration was done to the lower leaf of 15-d-old tomato plants. Seven days after infiltration the plants were moved to a greenhouse until the end of the experimental period, during which the plants were subjected to stress and subsequently allowed to recover. The relative humidity in the greenhouse ranged from 80% to 85% during the morning and from 40% to 50% at midday. The minimum and maximum temperatures were 18 °C and 28 °C, respectively. The maximum light intensity at midday was 1000 μE m^{-2} s^{-1}.

Imposition of low-moisture stress

For each experiment two subsets of plants were maintained. One set of plants from each treatment (wild type, infiltrated only with pTRV2 mock and pTRV2 *lea4*) was maintained at 100% field capacity (FC) and stress was imposed to the other set of plants.

The plants were maintained at 100% FC until imposition of moisture stress. Moisture stress was imposed by decreasing the soil water status gradually over 1 week until it was reduced to 50% of FC, and the plants were maintained at this stress level for 1 week. Soil moisture regimes were monitored gravimetrically by weighing the pots three times a day. At a given moisture regime, the exact soil water potential was assessed using a WP4 dewpoint potentiometer (Decagon Devices Inc, Washington, DC, USA). At the end of the stress period, moisture stress responses were assessed by taking leaf samples from the uppermost fully expanded leaves of both stressed and non-stressed plants.

RT-PCR: Total RNA was extracted from the newly developing leaves of tomato plants, except the Agro culture-infiltrated ones, as described by Datta *et al*. (1989). RT-PCR was performed using primers of pTRV-2 (forward: 5′-CTT TAT TAT TAC GGA CGA GTG GAC-3′; reverse: 5′-ACC CCT TTT GCC TTT GTA ACC-3′) to ensure viral spread in the system.

Northern analysis: Northern blot hybridizations were carried out according to Sambrook and Russell (2001). RNA (20 μg) from each sample was separated in formaldehyde denaturing gel and transferred to nitrocellulose Hybond membrane and fixed by exposure to UV (1200 μJ for 60 s) in a UV cross-linker. The blot was probed with the respective inserts prepared using either PCR or random labelling. Pre-hybridization was done at 42 °C for 2 h and hybridization was done at 55 °C overnight with blocking solution (0.5 M Na phosphate buffer, 1 mM EDTA, and 7% SDS) having a pH of 7.2. Probes were prepared by PCR or random labelling of the respective gene fragments with [^{32}P]dCTP (3000 Ci $mmol^{-1}$). The hybridized blot was washed in 2× SSC containing 0.1% SDS (w/v) twice. A further blot was washed in the sequence with 2× saline sodium citrate (SSC) for 10 min at 37 °C, 6× SSC for 10 min at 37 °C; 4× SSC for 10 min at 37 °C; 2× SSC for 15 min at 55 °C; 0.2× SSC for 10 min at 55 °C. The washed blot was exposed to Kodak X-ray film overnight (at –70 °C) and developed by autoradiography.

Dot blot analysis: Protein (50 μg $spot^{-1}$) was spotted on the nitrocellulose membrane. After drying, blots were blocked with 4% casein for 1 h at 37 °C in the rocker and subsequently probed with primary antibody at 2:1000 dilutions in casein (Savitha, 2000; Bindu, 2003). The blots were washed and developed after incubating with secondary antibodies as described earlier.

Western blot

Protein extraction: Tomato leaf material collected from silenced, mock-infiltrated, and wild-type plants was frozen in liquid nitrogen and ground in 100 mM TRIS-HCl buffer (pH 7.8) containing PMSF (1 mM) and benzamidine (5 mM) at 4 °C, and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was used for further experiments.

Preparation of the heat-stable protein fraction: The heat-stable protein fraction was prepared for the LEA4 blot by incubating total soluble protein in boiling water for 10 min and centrifuging at 10 000 *g* for 10 min at 4 °C. The clear supernatant containing soluble protein at 100 °C was the heat-stable fraction (Uma *et al*., 1995; Jayaprakash *et al*., 1998).

Protein (80 μg $lane^{-1}$) was run on SDS-PAGE gel and electro-blotted onto a nitrocellulose membrane according to Khyse-Andersen (1984). Blots were blocked using 4% casein in PBS for 12 h at 4 °C and probed with groundnut LEA4 primary antibody raised in rabbit. Further, blots were incubated with alkaline phosphatase-conjugated IgG (1:1000 dilutions) for 1 h at room temperature and developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrate (Chang *et al*., 1999; Duncan and Widholm, 2004).

Assessing the stress damage

Leaf water relations: Fully expanded leaves from stressed and well-watered tomato plants were sampled at midday, quickly sealed, and kept on ice. One set of five leaves was used to determine the leaf relative water content (RWC) and the other was used to determine leaf osmotic potential (Ψs). Leaf samples collected in a similar manner were used for determining other biochemical parameters.

Relative water content: After determining the fresh weight, the leaf segments were floated on deionized water for 5 h to determine their turgid weight. The dry weight was determined after oven-drying to a constant weight. The RWC was calculated using the formula:

$$\text{RWC}\% = 100\times[(\text{fresh weight}-\text{dry weight})/(\text{turgid weight}-\text{dry weight})]$$

Leaf solute potential (Ψs): Leaf samples were frozen in liquid nitrogen, thawed, and centrifuged for 5 min at 20 000 *g*. The Ψs of the extracted sap was measured by VAPRO vapour pressure osmometer (Wescor Inc., Logan, UT, USA).

$$\Psi S_{100} = (\Psi_S \times \text{RWC})/100$$

Osmotic adjustment: From the values of RWC and solute potential of control and stress-grown plants, the osmotic adjustment was calculated using the formula:

$$\text{Osmotic adjustment (OA)} = \text{drought leaf } \Psi_{S100} - \text{irrigated leaf } \Psi_{S100}$$

The protocol used here was adapted from Flower and Ludlow (1986) and Subbarao *et al*. (2000).

Chlorophyll estimation: Chlorophyll extracted from 100 mg of leaf tissue in an acetone:DMSO mix was centrifuged and the supernatant was made up to a known volume. The absorbance was recorded at 663 nm and 645 nm using a UV-Visible spectrophotometer (UV 2450; Shimadzu). Total chlorophyll was estimated (Hiscox and Israelstam, 1979; Nageswararao *et al*., 2001) and the percentage reduction over control was calculated.

TTC assay: The TTC (2,3,5-triphenyl tetrazolium chloride) assay was done to measure the extent of cell viability. TTC solution was prepared by dissolving 0.4% TTC (Sigma Aldrich, India) in 50 mM sodium phosphate buffer (pH 7.4). Leaf segments (1 cm diameter) were prepared from the fully expanded third leaf from the top of well-watered and moisture-stressed plants and incubated in TTC solution at room temperature for 5 h while being shaken. The leaf segments were washed to remove unbound formazan and boiled with 5 ml of 2-methoxy ethanol until dry to extract the bound TTC. The absorbance was measured at 485 nm using a UV-2450 UV visible spectrophotometer (Shimadzu) (Towill and Mazur, 1975; Senthil-Kumar and Udayakumar, 2004).

XTT assay: The superoxide radicals in stress and control leaves were quantified by XTT [(sodium 3′ [1-(phenylamine-carbonyl-3, 4-tetrazolium)]-*bis* [4 methoxy-6-nitro] benzene sulphonic acid hydrate] assay by adopting a previously described protocol (Sutherland and Learmonth, 1997; Able *et al*., 1998; Schopfer *et al*., 2001). Leaf segments (1 cm diameter) were incubated in 1 ml of medium containing 100 μM XTT and 25 μM of PMS (*N*-methyl dibenzopyrazine methyl sulphate) in 20 mM potassium phosphate buffer pH 6.0 for 6 h. The extent of reduction of XTT was assessed to quantify the chromophore formed using a micro-titre plate (Sunrise-Magellan, Tecan, A-5082, Australia) at absorbance of 470 nm.

Response of leaf segments to oxidative stress

The excised leaves from wild-type and silenced plants maintained at 100% FC were used to assess the oxidative-stress response. Leaf segments were subjected to oxidative stress by incubation in the media containing 3 μM methyl viologen by initially exposing them to an induction light intensity of 800 $\mu E\,m^{-2}\,s^{-1}$ for a period of 6 h and then to high light at 1800 $\mu E\,m^{-2}\,s^{-1}$ for 2 h. The extent of superoxide radical generation was assessed by XTT assay at the end of the stress period.

To study membrane leakage, leaf segments at the end of stress were floated on deionized water for 3 h and the extent of electrolyte leakage into the bathing medium was recorded using a conductivity meter (Elico-India, CM183, EC-TDS analyser). Subsequently, the leaf segments were boiled for 30 min and allowed to cool. The final reading was recorded and the loss of membrane integrity was determined using the formula

$$\text{Percentage leakage} = \{(\text{final reading} - \text{initial reading})/\text{final reading}\} \times 100$$

This protocol is modified from Leopold *et al*. (1981) and Tripathy *et al*. (2000).

Results

Silencing the tomato pds *gene*

Tomato plants infected with pTRV2 *tompds* developed photo-bleaching symptoms on the upper leaves 20 d post-Agro-infiltration and the symptoms continued to appear in the leaves which subsequently developed even after 30–35 d (Fig. 1). Endogenous transcript levels of *pds* were assessed by northern analysis using the *pds* probe from tomato. In the pTRV2 *tompds*-infiltrated plants, a marked reduction in *pds* transcript levels was seen by 15 d post-infiltration and no transcripts were detected after 20 d (Fig. 2).

To assess the extent of down-regulation of the endogenous *pds* gene by heterologous probes from *Nicotiana benthamiana* and groundnut, the tomato plants were infiltrated with *Nicotiana benthamiana pds* (*nbpds*) and groundnut *pds* (*gnpds*). In the plants infiltrated with *nbpds*, marked photo-bleaching was seen similar to those plants silenced by *tompds* (Fig. 1), though the extent and efficiency of silencing of *pds* was marginally less. In *tompds*-silenced plants, the chlorophyll content decreased by 66%, whereas with *nbpds* the reduction in chlorophyll was only 56% as compared with mock-infiltrated plants (Fig. 3). Even with *gnpds* silencing a significant reduction in chlorophyll content was seen, though the reduction was only 20% less compared with *tompds*-silenced tomato plants (Figs 1, 3). These results signify the efficiency of a TRV-based VIGS system in tomato and they also suggest that heterologous probes can be used to silence the gene of interest.

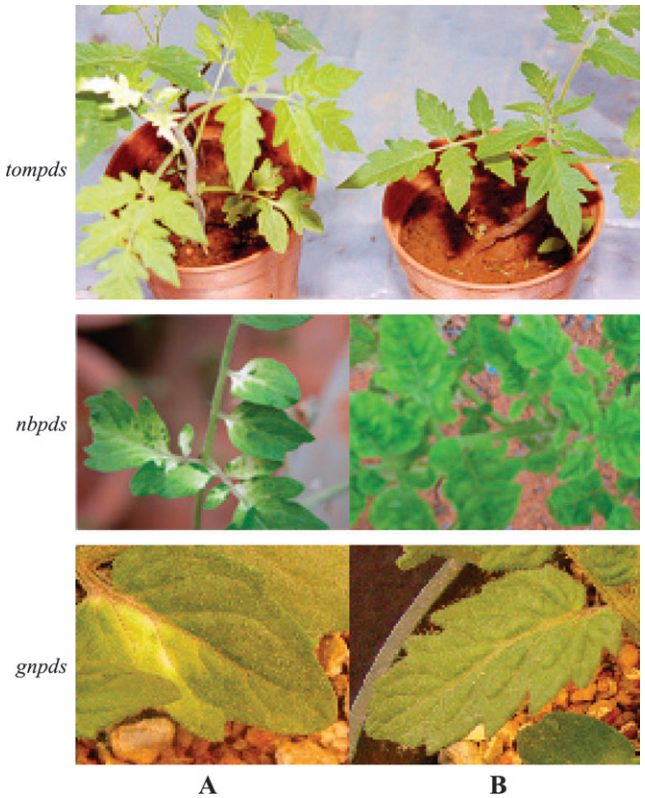

Fig. 1. Endogenous *pds* gene silencing in tomato using *pds* fragments from tomato (*tompds*), groundnut (*gnpds*), and *Nicotiana benthamiana* (*nbpds*). Fifteen-day-old tomato (Arka vikas or Micro tom) plants were infiltrated with pTRV2-carrying tomato or *Nicotiana benthamiana* or groundnut *pds*: (A) *pds*-silenced photo-bleached plant; (B) mock-infiltrated plants. The photographs were taken 25 d after infiltration.

Relevance of the lea4 *gene*

Cloning of lea4: The functional significance of *lea4*, an ABA-responsive stress gene cloned from groundnut, was examined in tomato. From the subtracted stress cDNA library constructed in groundnut (*Arachis hypogaea*) a 400 bp gsi87 clone (Gopalakrishna, 2001) was selected for the present studies. Besides, with gsi22 (groundnut putative *lea4*) the gsi87 cDNA sequence showed homology with the seed maturation protein LEA4 of *Glycine max*, *Glycine soja*, and *Glycine tomentella*, indicating that gsi87 belongs to the *lea4* group of genes.

Infection and spread of pTRV vectors in tomato: Fifteen-day-old tomato plants were infiltrated with pTRV2 (referred to as mock) or pTRV2 *lea4*. A separate set of control plants was also maintained. To assess the infection and subsequent spread, RNA was extracted from non-infiltrated upper leaves and the expression of the transcript was assessed by RT-PCR with specific primers of pTRV2. The presence of pTRV2 *lea4* and pTRV2 was seen on the fifth and seventh day post-Agro infiltration. However, in the wild-type plants no signal was found (Fig. 4).

Stress imposition: Prior to stress imposition, the plants were grown at 100% FC (–0.0312 MPa). A separate set of plants infected with pTRV2 *tompds* was maintained as a positive control to assess the time taken for spread. Stress was imposed on the pTRV2 *lea4*, mock-infiltrated, and wild-type plants when visual symptoms of photo-bleaching were noticed in the pTRV2 *tompds* plants. The soil moisture status was gradually decreased to 50% of field capacity (–1.6 MPa) and maintained at this stress level for 7 d (see Materials and methods for details). After the stress period, plants were rewatered to study the recovery response. At the end of the stress period and after recovery, the plants were analysed for the stress effects. In all the treatments a separate set of well-watered plants was also maintained.

The lea4 transcripts were low in silenced plants: In wild-type plants *lea4* expression was seen even under non-stress conditions, but the expression was much higher under stress. A substantial reduction was seen in *lea4*-silenced plants under both non-stress and stress conditions. No reduction in *lea4* transcripts was observed in mock-infiltrated plants (Fig. 5). However, other known stress-responsive genes like *apx* and *elip* or constitutively expressed genes like *rbcs* and *pds* showed similar expression levels in both the silenced and control plants.

LEA4 protein level: The extent of reduction in the LEA4 protein level was examined by dot blot analysis. Several

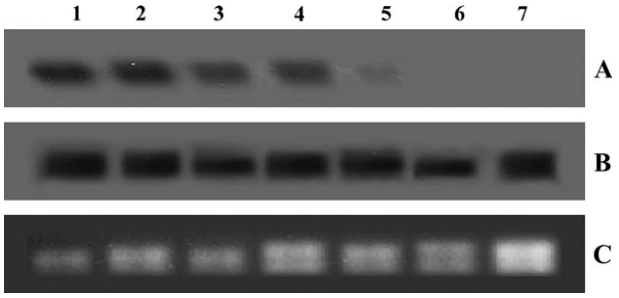

Fig. 2. Northern blot showing the reduction in endogenous *pds* transcript levels in tomato. In *tompds*-silenced plants, RNA was isolated on the 7th (3), 10th (4), 15th (5), 20th (6), and 25th (7) day after infiltration. RNA from wild-type (1) and mock-infiltrated (2) plants was isolated on the 15th day after infiltration. RNA was blotted onto the nitrocellulose membrane and probed with *pds* from tomato (A) and *rbcs* from groundnut (B). (C) A representative blot of 18s RNA showing equal loading of RNA. 1, Wild-type; 2, mock-infiltrated; 3–7, pTRV2 *tompds*-infiltrated plants.

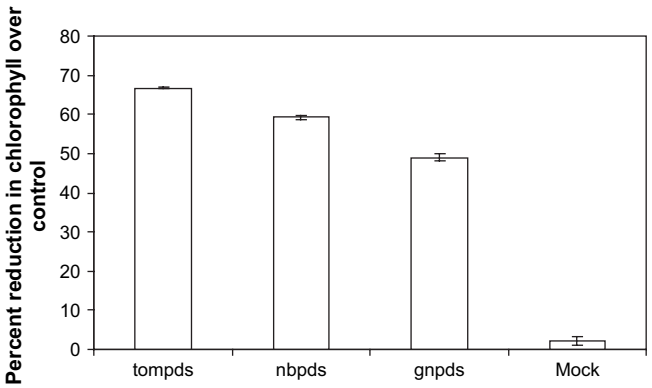

Fig. 3. Chlorophyll content in the *pds* gene-silenced tomato. Lower leaves of tomato plants were infiltrated either with pTRV2 *tompds* or pTRV2 *nbpds*. Thirty days after infiltration the chlorophyll content in the silenced third newly developed leaf was estimated. The values are the mean of three independent experiments. Bars represent the standard error.

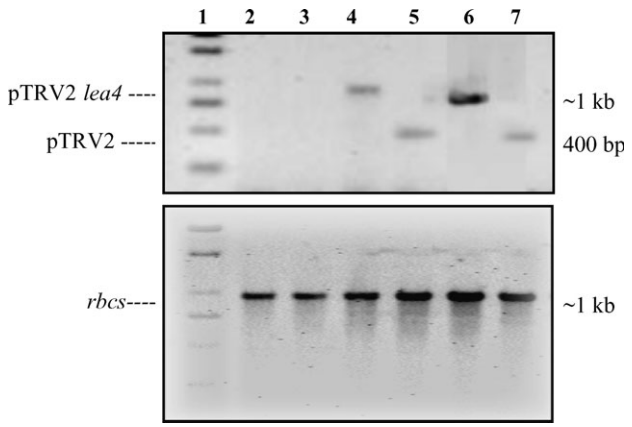

Fig. 4. RT-PCR showing the presence of viral transcripts in infiltrated plants. Lower leaves of 15-d-old tomato plants were infiltrated with pTRV2 or pTRV2 *lea4*. On the 5th and 7th days after infiltration, RNA was extracted from the third newly developed fully expanded leaves and RT-PCR was performed. Lanes: 1, 1 kb gene ruler (MBI Fermentas, USA); 2 and 3, wild-type; 4 and 6, pTRV2 *lea4*; 5 and 7, mock-infiltrated plants; 3, 4, and 5, 5 d after infiltration; 6 and 7, 7 d after infiltration. The lower panel represents the amplification of the *rbcs* gene from the corresponding cDNA pools mentioned in the top panel as a positive control to depict equal loading of cDNA.

stress-responsive proteins like LEA3, LEA2, HSP90, and HSFA showed higher accumulation in the silenced, mock-infiltrated, and wild-type plants under stress. An increase in LEA4 protein levels was seen in wild-type and mock-infiltrated plants under stress. The protein levels of HSP104, RuBisCO, and LEA1 remained the same in the silenced plants under stress and non-stress conditions. However, the level of antibody raised against the DNA-binding domain of HSFA (HSF DBD) and ferritin increased in the silenced plants under stress, whereas a marginal decrease of HSF DBD was seen in mock-infiltrated and wild-type plants (Fig. 6). Western analysis also confirmed that LEA4 protein levels were substantially reduced in silenced plants under both stress and non-stress conditions, whereas the RuBisCO LS protein levels were the same in both wild-type and silenced plants (Fig. 7). Both northern and western analysis clearly indicated the marked decline in *lea4* transcripts and protein in the pTRV2 *lea4*-infiltrated plants.

Stress responses of lea4*-silenced plants*

Phenotypic symptoms: Typical wilting of leaves was observed in pTRV2 *lea4*-silenced plants, whereas wild-type and mock-infiltrated plants did not wilt at the same stress levels (Fig. 8A). Further the mock-infiltrated and wild-type plants on rewatering recovered faster compared with the silenced plants (Fig. 8B). Wilting symptoms were observed in all the three experiments conducted, even with marginal variation in light intensity, temperature, and relative humidity during the experimental period.

Osmotic adjustment was reduced in silenced plants: The stress imposed in this study was gradual, to bring about adequate osmotic adjustment for adaptation. The osmotic adjustment of wild-type and mock-infiltrated plants was substantially higher under stress, whereas *lea4*-silenced plants showed less osmotic adjustment. Even after being brought back to 90% of field capacity by rewatering, there was no significant change in osmotic adjustment in *lea4*-silenced plants (Fig. 9A).

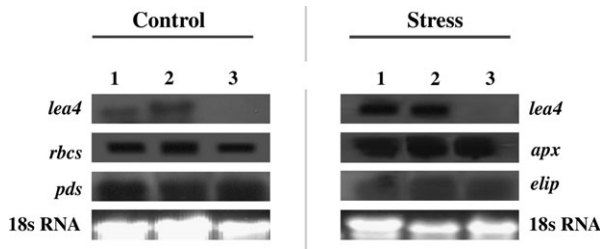

Fig. 5. Northern blot showing the silencing of endogenous *lea4*. RNA was extracted from the third newly developed fully expanded leaves of wild-type (1), mock-infiltrated (2), and pTRV2 *lea4*-infiltrated (3) plants. An RNA blot prepared from non-stressed plants was probed with *lea4*, *rbcs*, and *pds*; a blot from stressed plants was probed with *lea4*, *apx*, and *elip*. Stress was imposed as described in Materials and methods. The probes for the *lea4*, *rbcs*, and *elip* genes are from groundnut, and the *apx* gene is from tomato.

Cell viability and superoxide radical levels under stress in lea4*-silenced plants*: Generation of reactive oxygen species and a decrease in cell viability are the primary responses of stress. Accumulation of superoxide radical was quantified by assessing the extent of reduction of XTT (Schopfer *et al*., 2001) and the cell viability by TTC reduction in the plants subjected to severe moisture stress. Although the cell viability decreased under stress in all the plants, the extent of reduction was more pronounced in *lea4* gene-silenced plants compared with wild-type or mock-infiltrated plants (Fig. 9B). The *lea4* gene-silenced plants showed the same

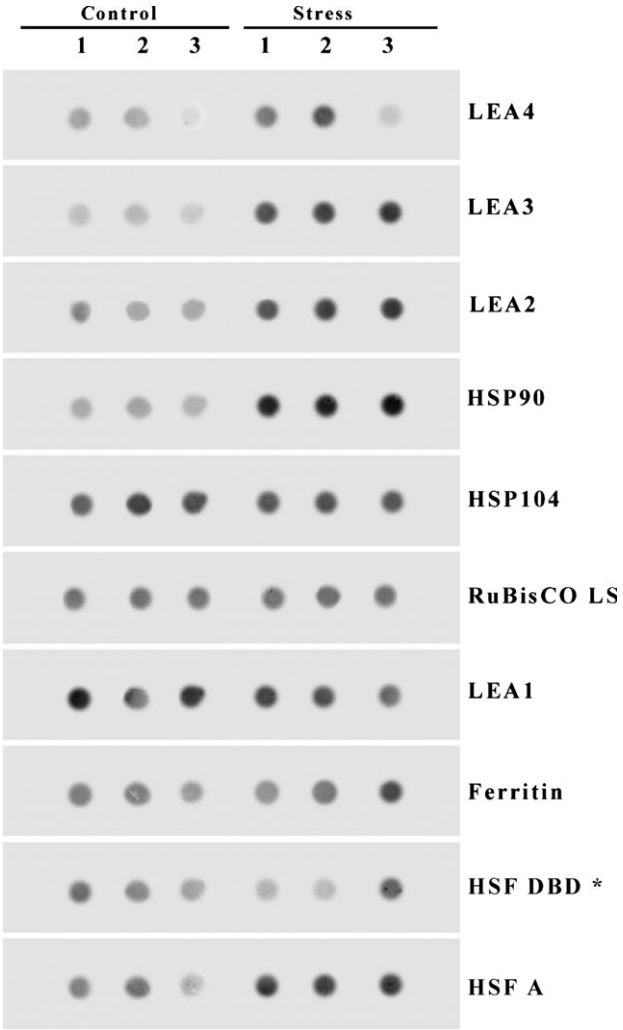

Fig. 6. Expression pattern of LEA4 and other stress-responsive proteins in silenced plants under stress. Protein extracted from the top, third fully expanded leaves of wild-type (1), mock-infiltrated (2), and pTRV2 *lea4*-infiltrated (3) plants were blotted onto the nitrocellulose membrane using a dot-blot apparatus, and probed using different antibodies. The stress protocols are the same as those mentioned in Materials and methods. HSP90 and HSP104 were kindly provided by Anil Grover, Delhi University, New Delhi. HSF DBD*, Antibody raised against the DNA-binding domain of HSFA; HSF, heat shock transcription factor antibodies raised against 16-mer sequence of YQQQQQSTDNQLQNQK (Senthil-Kumar *et al.,* 2003); LEA, late embryogenic abundant; HSP, heat shock protein; RuBisCO LS, large subunit of RuBisCO.

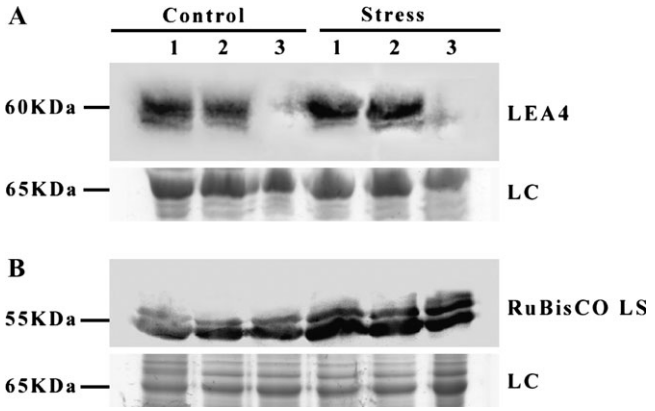

Fig. 7. Western blot showing the down-regulation of LEA4 protein levels in the silenced tomato plants. Samples were collected from the top, third fully expanded leaf of wild-type (1), mock-infiltrated (2), and pTRV2 *lea4*-infiltrated (3) plants. Proteins were resolved on 10% SDS-PAGE and probed using antibodies raised against either LEA4 (A) or RuBisCO large subunit (B). The lower blot in each panel represents the CBB-stained SDS-PAGE gel to show the equal loading of protein. The stress protocols are same as those mentioned in Materials and methods. LC, Loading control.

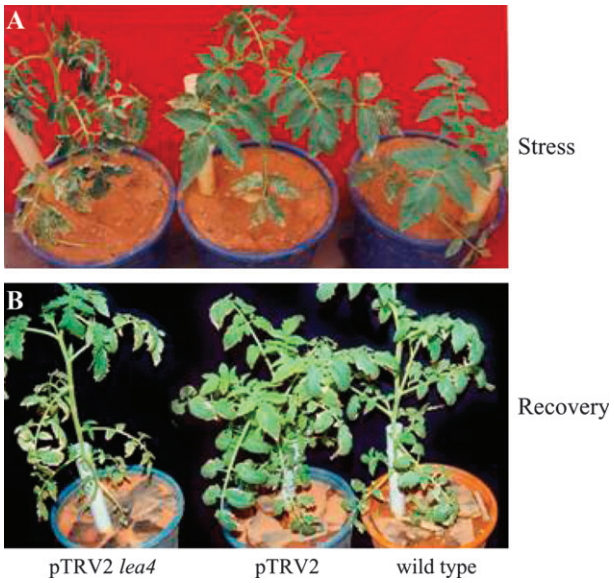

Fig. 8. Stress response of *lea4* gene-silenced plants. The photographs of pTRV2 *lea4*, mock-infiltrated, and wild-type plants were taken at the end of stress (A) and 5 d after recovery (B). A gradual low-moisture stress was imposed and, after reaching 50% FC, the plants were maintained at this stress level for 1 week. Subsequently the plants were watered to 90% FC and allowed to recover for 5 d. The silenced plants were subjected to water deficit once. Three such independent experiments were conducted and the results were consistent.

level of superoxide radical as that of wild-type plants under non-stress, whereas under stress the silenced plants accumulated very high levels of free radicals (Fig. 9C).

Higher oxidative stress damage in lea4*-silenced plants*: The oxidative stress response was examined in control and *lea4*-silenced plants maintained under well-watered conditions.

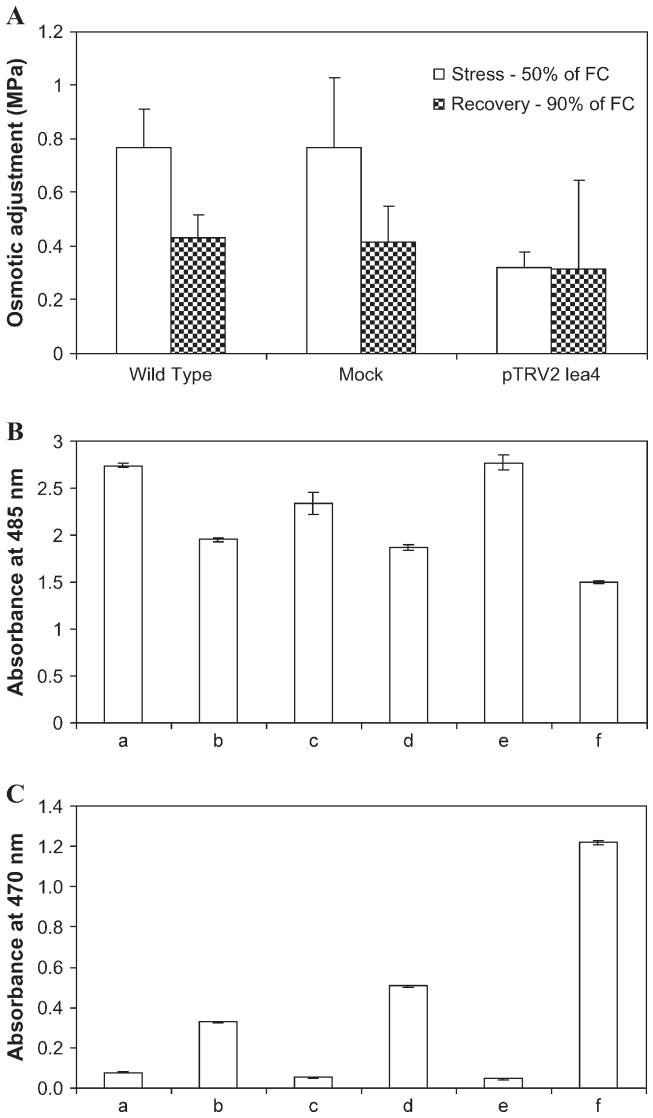

Fig. 9. Changes in osmotic adjustment, cell viability, and superoxide radical levels in lea4-silenced plants grown under stress. At the end of the stress period (as described in Materials and methods) and 4 d after recovery, leaf osmotic adjustment (A) was determined in wild-type, mock-infiltrated, and pTRV2 *lea4*-infiltrated plants. The extent of cell viability and superoxide radical levels was measured in the leaf segments collected from well-watered and stressed plants by TTC assay (B) and XTT assay (C), respectively. The values given here are the means of three independent experiments and the bars represent the standard error. (B, C) a, Wild-type well-watered; b, wild-type stress 50% FC; c, mock-infiltrated well-watered; d, mock-infiltrated stress 50% FC; e, pTRV2 *lea4* well-watered; f, pTRV2 *lea4* stress 50% FC.

Leaf segments collected from such plants were subjected to high-light, methyl viologen-induced oxidative stress for a relatively long time, sufficient to express the cellular protection machinery. Membrane damage was relatively high in the *lea4*-silenced plants compared with the mock-infiltrated plants (Fig. 10A). The silenced plants also showed enhanced susceptibility to oxidative stress by having 2-fold excess free radicals compared with wild-type and mock-infiltrated plants (Fig. 10B).

Discussion

Stress adaptation is complex and mechanisms involved in bringing about tolerance at the cellular level are quite diverse, tissue specific, and related to stress intensity or diversity. In these stress responses, expression of a number of stress-specific genes plays a crucial role. Transcriptome analysis using microarray technology has identified several genes that are induced by abiotic stresses. One group of these stress-specific genes encodes products that directly protect the plant cell against stresses, whereas the products of another group regulate gene expression and signal transduction during abiotic stress (Seki *et al*., 2001*a*, 2004; Chen *et al*., 2002; Vinocur and Altman, 2005). For many of these genes the annotation available is only based on the sequence similarities. Characterization of stress transcriptome and validation of their relevance is necessary to understand fully the diverse stress-adaptive mechanisms and to identify key regulatory genes (Shinozaki *et al*., 2003).

VIGS is a potential system for characterizing the functions of plant genes. Unlike T-DNA insertional mutants, VIGS does not require specific transformation protocol and hence it is a high-throughput technique. One of the specific advantages of VIGS is that cDNAs, which are not full length, can be efficiently characterized. Often it is considered that about 200–300 bp are adequate to induce efficient silencing (Burch-Smith *et al*., 2004).

Several recent reports describe silencing of a number of defence-signalling genes involved in disease resistance (Liu *et al*., 2002*b*) and genes involved in hormonal responses and metabolic processes (Burton *et al*., 2000; Liu *et al*., 2002*a*, *b*). However, there are no reports about the relevance of abiotic stress-response genes studied using VIGS.

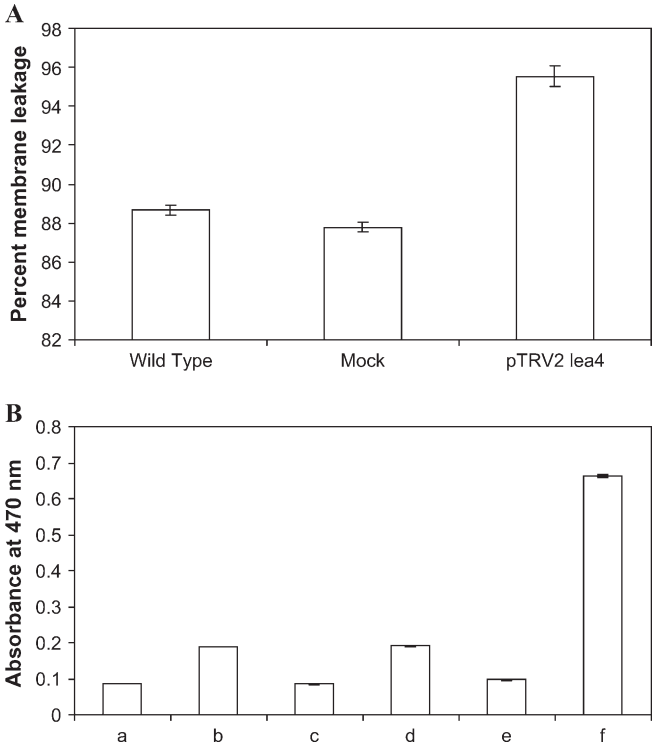

Fig. 10. Membrane damage and superoxide radical levels in the *lea4*-silenced tomato plants. Leaf segments were collected from well-watered wild-type, mock-infiltrated and pTRV2 *lea4*-infiltrated plants and were exposed to methyl viologen-induced high-light stress (1800 μE m^{-2} s^{-1}) for 6 h with prior induction to light stress (800 μE m^{-2} s^{-1} for 2 h). After 8 h of recovery, the extent of membrane damage was determined (A) and, immediately after the stress, superoxide radical levels were assessed by XTT reduction assay (B). The values given here are means of three independent experiments and the bars represent the standard error. (B) a, Wild-type non-stress; b, wild-type high-light stress; c, mock-infiltrated non-stress; d, mock-infiltrated high-light stress; e, pTRV2 *lea4* non-stress; f, pTRV2 *lea4* high-light stress.

Heterologous probe lea4 *from groundnut was effective in tomato*

Large-scale functional genomics screens have been undertaken with tobacco mosaic virus- and barley stripe mosaic virus-based vectors in *Nicotiana benthamiana* and barley (Holzberg *et al*., 2002). The functional significance of relevant genes can be studied using the VIGS approach only if efficient VIGS vectors are available for each species; however, the limitation is a lack of a wide range of specific VIGS vectors for different species. There are no specific VIGS vectors available for groundnut. Studies conducted with TRV-, potato virus-, pea early browning virus-, and white clover mosaic virus-based VIGS vectors using the *pds* gene from groundnut and from other legumes did not show any photo-bleaching in groundnut (data not given). There was no viral multiplication and spread observed when artificial infection was performed in groundnut and hence these vectors tried were not effective in silencing the native *pds* gene. Hence, the option was taken to silence the groundnut *lea4* gene in the tomato system where the efficiency of silencing by the TRV VIGS vector is relatively high. There are reports suggesting that adequate levels of silencing can be achieved by using probes from heterologous species. The *pds* gene fragment derived from a monocot silenced the endogenous gene in *Nicotiana benthamiana* in spite of the remote evolutionary relationship between these two species (Benedito *et al*., 2004). However, when heterologous probes were used, the extent of silencing and duration of silencing were less when compared with the probes from the host species, for example, a *pds* fragment from tomato silenced the endogenous *pds* in *Nicotiana benthamiana* with a very high level of photo-bleaching, as compared with the *pds* gene fragment from lily (Benedito *et al*., 2004).

Even in this study a fairly high degree of photo-bleaching was observed when heterologous probes from *Nicotiana benthamiana* (*nbpds*) and groundnut (*gnpds*) were used to silence endogenous *pds* of tomato (Fig. 1). The nucleotide identity between partial cDNA of *gnpds* and full-length *tompds* was only 83%, whereas it was 92% between the partial *nbpds* sequence and the full-length *tompds*. In spite of this, the extent of *pds* gene silencing in tomato using *gnpds* was relatively high, suggesting that the

heterologous probes from groundnut can be used to silence the target gene in tomato.

Initially, the extent of cross-reaction of *lea4* probe from groundnut in tomato was examined by dot-blot analysis. There was a significant cross-reaction indicating a fairly high degree of homology (data not shown). Detection of signal in northern analysis of tomato transcripts using *lea4* from groundnut also confirmed this aspect (Fig. 5). Antibodies raised against the LEA4 peptide cross-reacted with tomato LEA4 (Figs 6, 7). The partial cDNA (581 bp) recently cloned (NCBI accession number DQ256365) from tomato also showed a nucleotide identity of 87% with gsi87 and 94% with gsi22.

Development of optimum moisture stress protocols

Stress-responsive genes are induced only upon exposure to stress. Under field-grown conditions stress occurs gradually, hence stress effects are often observed only after plants experience a reduction in tissue water levels, which may take a few days to weeks after the imposition of stress. Hence the relevance of stress genes can best be assessed when the period of silencing of the stress gene coincides with defined stress levels.

VIGS is transient and reversal of silencing occurs after a few weeks of infiltration (Liu *et al*., 2002*a*). So it is necessary to assess the time of initiation of the silencing process and its duration. Using *pds* gene silencing as a reporter system in tomato, the initiation of the silencing symptom, its duration, and reversal time were examined. The results showed that silencing can be achieved 20 d after infiltration, and duration of silencing was up to 35 d (data not given). Subsequently, in all the present experiments, a parallel subset of the plants silenced with the *pds* gene were maintained to serve as a reporter system. Stress was imposed in the pTRV2 *lea4*, mock-infiltrated, and wild-type control plants 3 d after photo-bleaching was observed in *pds*-silenced plants. By monitoring the soil water status, stress was gradually imposed so that the plants experienced the desired stress levels (50% FC) in 7 d and, subsequently, the plants were maintained at this stress level for another week. The plants therefore experienced defined stress levels for 22 d after infiltration. The *lea4* transcript levels quantified 5 d after stress imposition confirmed that there was a reduction in transcripts, indicating that the effective silencing period coincided with the stress period.

Silenced plants showed a decline in *lea4* transcript levels, whereas the expression levels of other stress-responsive genes studied (*apx* and *elip*) and constitutively expressed genes like *rbcs* and *pds* were not affected. Western analysis also confirmed the decline of LEA4 protein levels, whereas the levels of other stress-responsive proteins like LEA3, LEA2, LEA1, HSP90, HSP104, and HSFA and non-stress-responsive proteins like RuBisCO large submit were unaffected. This clearly indicated that the desired phenotype with reduced *lea4* transcript levels was obtained to assess the stress responses.

Performance of lea4*-silenced plants under stress*

LEA proteins that accumulate during the later stages of embryogenesis are implicated in the desiccation tolerance of embryos. Many LEA proteins respond to ABA and are also expressed under osmotic stress and at low temperatures. Their predicted function has been fairly well elucidated (Baker *et al*., 1988; Ganeshkumar, 1999; Kaur and Gupta, 2005). LEAs protect the cellular structure from the effect of water loss by acting as hydration buffer, sequestering ions, renaturing unfolded proteins, and protecting other proteins (Bray, 1993; Kaur and Gupta, 2005). The majority of LEA proteins are highly hydrophilic; however, LEA4 is slightly hydrophilic and possibly contains an amphipathic alpha helix (Dure, 1993; Chandrababu *et al*., 2004; Goyal *et al*., 2005). Group-4 LEA proteins contain a conserved N-terminal domain that forms an alpha helix. These are rich in glycine and amino acid-containing hydroxyl groups, forming unsaturated random coils (Dure, 1993). They bind water molecules and may also act as reverse chaperones, thus stabilizing the surface of membranes and possibly they protect by binding water molecules, functioning as a solvation film. Expression of the LEA4 group of proteins is shown to be in response to drought, salinity, ABA, and low temperatures (Cohen *et al*., 1991). Earlier studies have indicated that the LEA group of proteins are highly expressed upon desiccation in mesophyllic tissues as well as in embryos of groundnut (Ganeshkumar, 1999; Savitha, 2000). Overexpression of the *le25* gene from tomato belonging to the LEA4 group led to an increased resistance to high salinity and freezing tolerance in yeast (Imai *et al*., 1996).

The *lea4* gene has been studied (Savitha, 2000; Bindu, 2003) and cloned from mature embryos (Dure, 1993), while *lea4* was previously cloned from groundnut leaves experiencing stress (Gopalakrishna, 2001; Gopalakrishna *et al*., 2001). High levels of expression of this protein in groundnut under stress suggested that *lea4* might be relevant in ameliorating stress effects (Sales *et al*., 2000; Savitha, 2000; Zang, 2000; Bindu, 2003). The present study provides evidence to show that the *lea4* gene plays a role in imparting desiccation-stress tolerance.

Under stress, *lea4*-silenced plants showed wilting symptoms at midday and the RWC was significantly lower than the mock-infiltrated plants. The silenced plants had less osmotic adjustment (Fig. 9A) and, hence, pressure potential was less, leading to visible wilting symptoms (Fig. 8A). The total osmolyte levels were also less in silenced plants. Even the recovery growth was poor on stress alleviation in silenced plants (Figs 8B, 9A). Several protective proteins, most notably LEAs (Ingram and Bartels, 1996), binding proteins (BiP) (Alvim *et al*., 2001), and ASR proteins (Yang *et al*., 2005) are well known to accumulate in

response to a decrease in tissue water content (Verslues *et al*., 2006). In view of the known function of LEA proteins, it is intriguing that *lea4* gene-silenced plants showed less osmotic adjustment and showed more wilting. Maintenance of a positive turgor and high water status in the cell has been reported in plants with enhanced expression of similar stress-responsive proteins like BiP (Alvim *et al*., 2001) and ASR (Yang *et al*., 2005), in addition to their major role as chaperones. *BiP* gene overexpressing transgenics have been shown to maintain cell turgor under stress (Cascardo *et al*., 2000; Alvim *et al.*, 2001). It is likely that increased osmotic adjustment by BiPs might have been one of the reasons for maintaining turgidity under dehydration stress, since the antisense BiP transgenic plants showed wilting symptoms and decreased osmotic adjustment under stress (Alvim *et al*., 2001). BiPs might also facilitate proper folding and maturation of the selected group of water stress-induced secretory proteins, which are involved in osmotic response mechanisms (Cascardo *et al*., 2000; Alvim *et al*., 2001). The LLA23, an ASR protein, also has similar structural motifs to those of LEAs and, hence, has relevance in imparting dehydration tolerance. In a recent study, overexpression of LLA23 maintained a higher tissue water status even up to 12 d of dehydration. Also, with a reduction in stomatal closure, LLA23-expressing transgenic plants had less water loss and maintained turgidity. It was attributed to the water-retaining ability of the LLA23 protein. However, the exact mechanism of these proteins in maintaining the water status of cells has not been shown. It is likely that the LEA family of proteins, which has similar function as BiPs and ASRs, may indirectly contribute to the osmotic adjustment and may play a role in cellular water retention under dehydration. However, detailed studies are essential to elucidate the exact function of LEA4 protein in regulating water relations of the cell under stress. Further, less cell viability (Fig. 9B), pronounced membrane leakage (Fig. 10A), and higher activity of superoxide radicals (Figs 9C, 10B) in *lea4*-silenced plants clearly indicates the direct involvement of the *lea4* gene in imparting tolerance under moisture-stress conditions.

In summary, it was demonstrated that heterologous probes could be used for VIGS if there is considerable sequence homology of the target gene in host species. Also it was shown that by developing suitable protocols for stress imposition, the functional relevance of stress genes can be efficiently studied by VIGS. However, the stress levels to which the plant is exposed should coincide with the maximum silencing period of the target gene.

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