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Expression of a unique plastid-localized heat-shock protein is genetically linked to acquired thermotolerance in wheat

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Abstract We have used a combination of molecular and classical genetic approaches to delineate the relationship between a specific HSP member and cell viability under heat stress. Using recombinant inbred lines (RILs) of wheat, derived from a cross of the thermotolerant cultivar 'Mustang' and the thermosusceptible cultivar 'Sturdy,' we have identified a unique HSP and a differentially expressed cDNA sequence, both related to the plastid-localized HSP26 gene family, that are closely associated with acquired thermotolerance in wheat. An isoform of HSP26 was synthesized under heat stress in all examined thermotolerant RILs and 'Mustang', and was absent in all examined thermosusceptible RILs and 'Sturdy.' Using a modified differential-display method, we have also identified a gene-specific cDNA sequence that is similar to other known members of the wheat HSP26 gene family and is selectively expressed in 'Mustang' and most of the examined thermotolerant RILs, but not expressed in 'Sturdy' and all the thermosusceptible RILs. These results suggest a genetic linkage between the acquired thermotolerance trait and the differential expression of a unique member of the HSP26 gene family.

Key words Stress proteins · Heat tolerance · Chloroplast · Genetics

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Introduction

When prokaryotic and eukaryotic organisms are exposed to a mild heat treatment, they acquire tolerance against a subsequent stronger heat shock that is normally lethal, exhibiting the well-known phenomenon of acquired thermotolerance (Hahn and Li 1990). Simultaneously, heat treatment elicits a so called heat-shock response leading to the immediate induction of a set of heat-shock proteins (HSPs) and their persistent expression over a time course at high temperature (Vierling 1991; Nguyen et al. 1992). The simultaneous occurrence of these two events suggests that HSPs may play a role in the process of thermotolerance acquisition.

Although HSPs are the best-studied stress proteins, with proven functions in cell protection, survival, and recovery in numerous species (Vierling 1991; Nguyen et al. 1992), there is limited evidence proving a cause-and-effect relationship between HSP induction and the acquisition of thermotolerance. Constitutive synthesis of heat-shock proteins in transgenic *Arabidopsis* plants with de-repressed activity of the heat-shock factor led to increased levels of thermotolerance (Lee et al. 1995). Similarly, constitutive expression of HSP70 in mammalian cells conferred thermoresistance (Angelidis et al. 1991). By contrast, constitutive expression of HSP70 in *Drosophila* did not affect thermotolerance and was, in fact, detrimental to growth at normal temperatures (Feder et al. 1992). Recently, HSP104 was shown to be important for the acquisition of thermotolerance in yeast (Sanchez and Lindquist 1990; Parsell et al. 1994), while HSP26 and HSP12 were not required (Petko and Lindquist 1986; Praekelt and Meacock 1990). Meanwhile, HSP30 has been shown to be essential for thermotolerance in *Neurospora crassa* (Plesofsky-Vig and Brambl 1995). These scarce and conflicting data cannot be readily extrapolated to other species due to species-specific differences in HSP production as well as the diversified molecular basis of acquired thermotolerance in different organisms. For example, the results of experiments with yeast, described above, cannot apply to *Drosophila melanogaster* as it does not express HSP104 while being able to acquire thermotolerance.

All HSPs belong to multigene families with the number of members ranging from 2 to 20 (Vierling 1991; Craig et al. 1987). Not all members of an HSP family may be required for cell

viability/thermotolerance, and different HSPs belonging to one class may exchange functions during the crisis period of heat stress, as demonstrated by the yeast HSP70 family (Craig et al. 1987). Moreover, in some circumstances, HSPs of different classes may substitute for the functions of each other, as was shown for yeast HSP70 and HSP104 (Sanchez et al. 1993). While HSP70 was found to be important for vegetative growth and HSP104 for thermotolerance in normal yeast cells, in a HSP104 deletion mutant of yeast, HSP70 became important for thermotolerance. Conversely, HSP104 plays a substantial role for vegetative growth under conditions of decreased HSP70 levels in HSP70 mutants.

Although high-temperature stress is a major factor leading to yield losses in several crop plants, we have a limited understanding of the molecular basis of their heat tolerance (Nguyen et al. 1992); and it is not known whether HSPs contribute to this trait (Nguyen and Joshi 1994). Obtaining single mutations is notoriously difficult in crop plants due to the complexity of their genomes. Since most HSPs belong to multigene families, reverse genetic experiments of knocking-out one or a few genes at a time are frequently not successful. Hence, traditional genetic experiments involving co-segregation analysis have tremendous potential to define HSPs that are important for thermotolerance in crop plants. To-date, no genetic association has been shown between the synthesis of any single HSP and acquired thermotolerance in plants (O'Connel 1994). Here we provide the first genetic evidence for the association between the expression of a unique member of the HSP26 gene family and acquired thermotolerance in wheat.

Our laboratory has previously identified two hexaploid winter wheat varieties, thermotolerant 'Mustang' and thermosusceptible 'Sturdy,' that differ in their capacity to acquire thermotolerance (Krishnan et al. 1989). Under heat stress, 'Mustang' synthesizes several unique low-molecular-weight (LMW) HSPs in the range of 16–30 kDa that are absent in 'Sturdy' (Krishnan et al. 1989; Weng and Nguyen 1992). We were, therefore, interested in examining whether these unique LMW HSPs are genetically associated with acquired thermotolerance in the F_6 progeny derived from the cross between 'Mustang' and 'Sturdy.' Here we report the possible genetic linkage between acquired thermotolerance and the differential expression of a unique member of the HSP26 gene family derived from the thermotolerant parent 'Mustang.'

Materials and methods

Plant material and thermotolerance measurements

The thermotolerant winter-wheat cultivar 'Mustang' and the thermosusceptible cultivar 'Sturdy' were used as parents to produce a single cross (Krishnan et al. 1989). We started a divergent selection program with a single F_2 population of 'Mustang' × 'Sturdy' to identify recombinant progenies with different levels of heritable acquired thermotolerance using the triphenyl tetrazolium chloride (TTC) cell-viability assay according to Chen et al. (Chen et al. 1982). We have produced F_6 recombinant inbred lines belonging to 40 families derived from divergent selection (20 F_2-derived thermotolerant families and 20 F_2-derived thermosusceptible families). Each family consisted of 6–10 lines which were advanced by the single-seed-descent breeding method. Several outstanding lines contrasting in thermotolerance were identified. A total of eight lines, including two parents, three thermotolerant RILs (#753, #774, and #794) and three thermosusceptible RILs (#619, #623, and #651), were selected for further molecular analysis. The selected thermotolerant F_6 RILs descended from the thermotolerant F_2 plants, while the thermosusceptible RILs were descended from the thermosusceptible F_2 plants.

For all experiments, we used 10-day-old seedlings grown in controlled conditions at 25°C with a 16-h photoperiod and a light intensity of 600 mmol $m^{-2}s^{-2}$ PPFD (RH 25–30%). To measure the thermotolerance of the F_6 RILs, we carried out a TTC cell-viability assay according to Chen et al. 1982) and Krishnan et al. (1989). Leaf segments of five seedlings belonging to the same line were bulked together to decrease the effects of variation due to continuing segregation in F_6 progeny.

In vivo protein labeling and two-dimensional electrophoresis

In vivo protein labeling by $^{[35]}$S-methionine incorporation total protein extraction, and one-dimensional (1-D) and two-dimensional (2-D) polyacrylamide-gel electrophoresis (PAGE) were performed as described by Vierling and Nguyen (1990) and Jorgensen et al. (1992). Dry gels were exposed with X-ray film (RM-1, Kodak) for 3–6 days to achieve a total of 3 million dpm × days. A molecular-weight-standard mixture (2-D SDS-PAGE standards, Bio-Rad) was separated on parallel 2-D gels that were stained with Coomassie R-250 dye (Sigma). Molecular weights and isoelectric-point (pl) values of the HSP26 isoforms were calculated based on the calibration curves derived from the separation patterns of standard proteins.

Development of polyclonal antisera

We constructed fusion plasmid pMAL-HSP26.6 using pJW102 DNA (Weng et al. 1991) (Genbank/EMBL accession number X34240) and a pMAL-CRI vector (New England Biolabs) and transformed it into *Escherichia coli* TB1 cells. Fusion maltose-binding protein (MBP)-HSP26.6 was expressed and purified following the manufacturer's instructions (New England Biolabs). The fusion protein was injected subcutaneously into a rabbit, and polyclonal antiserum was further purified on an affinity MBP-Sepharose 4B column according to the manufacturer's instructions (Pharmacia).

One-dimensional protein electrophoresis and immunoblotting

Total protein samples were prepared as in Hernandez and Vierling (1983) from 300 mg of leaf tissue after plants were exposed to 37°C for the defined periods. Sample aliquots containing 30 mg of protein were separated by 1-D PAGE according to Laemmli (1970). After 1-D or 2-D PAGE, gels were blotted onto a nitrocellulose membrane (Trans-Blot transfer medium) in the Bio-Rad Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions and subjected to the Western-blotting procedure according to the manufacturer's instructions (ECL kit, Amersham). A 1 : 100 dilution of primary antibodies and a 1 : 10 000 dilution of secondary antibodies (whole Ig, donkey anti-rabbit, Amersham) were used. For the detection of radioactive proteins and immunoblotting on the same membrane, the blots were first subjected to the immunoblotting procedure, then air-dried and exposed to the X-ray film as described above for the dry gels.

Nucleic-acid hybridization, differential display, DNA cloning, sequencing and sequence analysis

Total RNA was isolated by the guanidine hydrochloride extraction method from 1 g of seedling leaf tissue from the eight selected lines of wheat described earlier following a 37°C, 1-h heat treatment. After treatment of 50 µg RNA with 10 U of DNAse 1 (GenHunter Corporation), this RNA was used for differential display experiments according to Liang et al. (1993); Joshi et al. (1996). For use in differential display, we designed an HSP26 family specific primer (15-mer CGCAAGGTCATCGAC) encoding the amino-acid sequence RKVID which is conserved in all reported chloroplast-localized HSP genes including two cDNAs from wheat (Weng et al. 1991; Nguyen et al. 1993). This amino-acid sequence is found close to the carboxyl terminus of proteins and is followed by four terminal amino acids. Separation of cDNA fragments, autoradiography, cDNA extraction and re-amplification were done according to Joshi et al. (1996). cDNA fragments of interest were cloned in a pCR-Trap vector (GenHunter Corporation) following the manufacturer's instructions and sequenced using a Sequenase Version 2.0 kit (United States Biochemicals). The Genetics Computer Group (GCG) sequence-analysis-program package was used for DNA and the predicted protein sequence analysis. Differential expression of cDNA fragments was confirmed by Northern-blot analysis as described by Joshi et al. (1992).

Results

Selected F_6 RILs differ in acquired thermotolerance

The F_6 recombinant progeny is theoretically characterized by 96.9% homozygosity and, hence, is still segregating. Therefore, for the molecular analysis we selected only six F_6 lines contrasting in their capacity to acquire thermotolerance. Three selected thermotolerant lines (#753, #774, and #794) belonged to three initially thermotolerant families, while three thermosusceptible lines (#619, #623, and #651) belonged to three initially thermosusceptible families. The TTC cell-viability assay was used to analyse their capacity to acquire thermotolerance. All thermotolerant lines and the thermotolerant parent 'Mustang' acquired 49–56% cell protection after the acclimation treatment, while all thermosusceptible lines and thermosusceptible parent 'Sturdy' developed only 20–25% cell protection (Table 1).

Table 1 Thermotolerance of parental and F_6 lines determined by a TTC cell-viability test

Line	Percent of thermoprotection[a]	
	$\bar{x}$	*SD*
Mustang	52.4	15.3
753	49.3	14.4
774	56.3	6.9
794	53.5	7.8
Sturdy	25.6	5.7
619	21.5	2.0
623	23.6	3.2
651	21.9	1.8

[a] Mean and standard deviation of three independent experiments

HSP26 production in F_6 RILs

Earlier, wheat genotypes 'Mustang' and 'Sturdy' were shown to vary in patterns of HSPs in the molecular weight range 16–30 kDa that were synthesized *in vivo* under heat stress (Krishnan et al. 1989). Several 'Mustang'-specific HSPs of 16–40 kDa were observed after the *in vitro* translation of mRNA isolated from the heat-treated seedlings (Weng and Nguyen 1992). Two major heat-shock-induced polypeptides of about 22 kDa and pl 5.5 and 5.0 (designated HSP26-I and HSP26-II, respectively) were *in vivo* labeled in the leaves of 'Mustang,' while only the one with pl 5.5 (HSP26-I) was present in the leaves of 'Sturdy' (Fig. 1). The same polypeptides were hybridized with anti-HSP26 antibodies on Western blots (Fig. 1). The molecular weight of approximately 22 kDa is predicted for an HSP26 mature polypeptide after the deletion of the transit peptide of about 4–5 kDa during post-translational transport of the HSP26 precursor to the chloroplasts (Vierling 1991).

When subjected to a prolonged heat treatment of 37°C, the thermotolerant cultivar 'Mustang' accumulated HSP26-related proteins in its leaves faster, and to a higher level, than the thermosusceptible cultivar 'Sturdy.' Densitometric measurements of the immunoblot showed that, at each time point between 1 and 12 h of incubation at 37°C, the density of the band corresponding to HSP26 was approximately doubled in the seedlings of 'Mustang' compared to 'Sturdy' (Table 2).

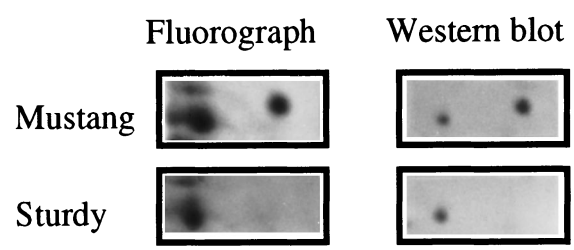

Fig. 1 HSP26 polypeptides in the leaves of 'Mustang' and 'Sturdy.' Total protein fractions from the seedlings of 'Mustang' and 'Sturdy' subjected to heat shock at 37°C for 2 h in the incubation medium supplemented with 50 mCi of ^{35}S-methionine were separated on two-dimensional PAGE. Immediately after electrophoresis, the gels were blotted onto nitrocellulose. On the left, autoradiograms from the blots are presented. On the right, the same blots were immunodetected using polyclonal antibodies against HSP26 from wheat using the ECL chemiluminescence detection system (Amersham). Regions of the gels containing HSP26 are presented. The basic end of the one-dimensional gels is on the left. Note the two isoforms of HSP26 (HSP26-I and HSP26-II) in 'Mustang' and one isoform (HSP26-I) in 'Sturdy'

Table 2 HSP26 accumulation in the leaves of 'Mustang' and 'Sturdy' during the time course of heat shock at 37°C. Total protein fractions were isolated, resolved on 1-D PAGE, and immunodetection was performed using polyclonal antibodies against the the wheat HSP26 and the ECL detection system (Amersham). Images on the X-ray films were scanned on a Molecular Dynamics Scanning Densitometer (Model 300B) and analyzed using Image Quant software

Cultivar and conditions of treatment			Volume of HSP26 band[a]	Percent of band volume[b]
Mustang	25°C		0	0
	37°C	0.5 h	3.9	0.5
	37°C	1 h	16.5	2.0
	37°C	4 h	257.7	30.1
	37°C	12 h	842.8	100
Sturdy	25°C		0	0
	37°C	0.5 h	5.1	0.6
	37°C	1 h	9.9	1.2
	37°C	4 h	137.1	16.9
	37°C	12 h	426.3	50.9

[a] Volume of the area on the background of X-ray films equal to a band area was subtracted from all band volumes
[b] Maximum band volume is 100%

In the leaves of F_6 RILs subjected to heat shock at 37°C for 2 h, we observed a recombination of all 'Mustang'-and 'Sturdy'-specific LMW HSP species. The majority of these HSPs were randomly distributed in thermotolerant and thermosusceptible lines (data not shown). The only exception was HSP26. Three thermotolerant RILs had both HSP26-I and HSP26-II polypeptides labeled in vivo after 2 h of incubation at 37°C, while three thermosusceptible RILs had only the HSP26-I polypeptide labeled under these conditions (Fig. 2). The difference between thermotolerant and thermosusceptible RILs was qualitative, since no trace of HSP26-II could be detected in thermosusceptible lines even when the gels were over-exposed (data not shown).

Thus, intriguingly, thermotolerant RILs expressed the same two HSP26 species (HSP26-I and HSP26-II) as the thermotolerant parent 'Mustang,' while the thermosusceptible RILs expressed only one HSP26 polypeptide (HSP26-I) as did the thermosusceptible parent 'Sturdy.'

Differential expression of HSP26 mRNA in selected F_6 RILs

Our protein studies suggested that differential expression of an HSP26 gene-family member is associated with thermotolerance in wheat. In order to study the differences in the mRNA populations among the F_6 RILs and parental lines in differential display experiments, we used a HSP26 gene-family specific primer which we designed based on the sequences of all plastid-localized plant HSP genes reported to-date. This

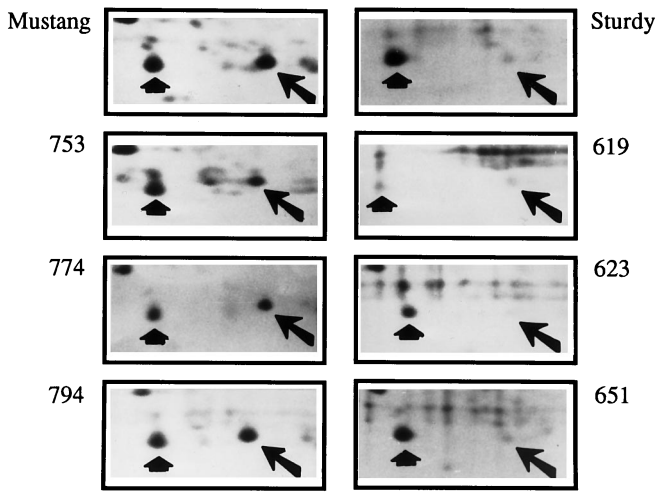

Fig. 2 Two-dimensional analysis of *in vivo* (Nguyen et al. 1994) ^{35}S-methionine-labeled proteins of selected F_6 lines and parental genotypes after 2 h of heat shock at 37°C. Lower portions of the gels are presented. The basic end of the one-dimensional gels is on the left. *Numbers* of RILs are given adjacent to corresponding fluorograms. Molecular weights and positions of markers are in kilodaltons. The HSP26-I polypeptide present in all lines is denoted by a *thick arrow*; the HSP26-II polypeptide, which is differentially expressed, is marked by a *thin arrow*

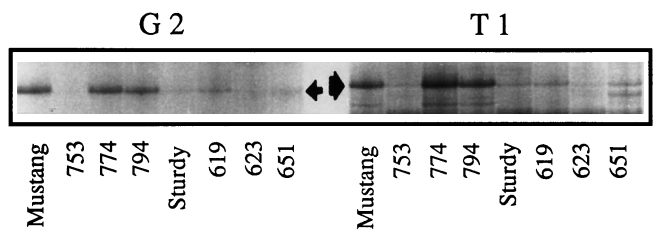

Fig. 3 Differential display patterns of total RNA from parental lines Mustang and Sturdy and six F_6 RILS, including three thermotolerant lines (#753, #774, and #794) and three thermosusceptible lines (#619, #623, and #651), after a heat shock of 37°C for 1 h. An HSP26.6 gene-family specific primer and two 3-anchored oligo-dT primers, namely T12VG (designated by *G*) and T12VT (designated by *T*), were used for PCR amplification from the total RNA isolated from seedlings. The differentially expressed fragments HSP26G2 and HSP26T1 are indicated by *arrows*

allowed us to target only cDNA sequences that were highly homologous to HSP26 genes and, specifically, the 3′ noncoding regions of these sequences. Using anchored oligo-dT primers T12VG and T12 VT, we observed two cDNA fragments (named G2 and T1) amplified in three out of four thermotolerant lines, namely, 'Mustang,' #774, and #794 (Fig. 3). These fragments were weakly expressed, or not detectable, in line #753, 'Sturdy,' and all three thermosusceptible lines. The two cDNA fragments, G2 and T1, were isolated from sequencing gels, further amplified, and used as probes for Northern-blot analysis with the samples of total RNA extracted from wheat seedlings of

eight selected lines after heat stress at 37°C for 1 h. We observed a clear differential expression of mRNAs homologous to cDNA G2. Similar to the differential-display results, the three thermotolerant lines, 'Mustang,' #774 and #794, strongly hybridized with the G2 fragment; whereas, thermotolerant line #753 and thermosusceptible lines 'Sturdy,' #619, #623, and #651 showed a very weak hybridization (Fig. 4). This observation was repeated by using a T1 fragment as a probe in a Northern blot (data not shown). Thus, using the differential-display method, we have isolated a gene-specific 3′ region of a unique member of the HSP26 gene family that is expressed during heat stress in three out of four thermotolerant lines, and which is absent, or weakly expressed, in all four thermosusceptible wheat lines.

Sequencing of the differentially expressed HSP26-related cDNA fragments

After cloning in the pCR-Trap vector, the fragments G2 and T1 were sequenced (Genbank/EMBL accession numbers L41503 and L41504). Multiple alignment of these sequences with homologous regions from two previously reported members of the HSP26 gene family from wheat is shown in Fig. 5. The G2 and T1 sequences are identical to each other except at four base pairs, and G2 is longer than T1 by 10 bp when the poly(A) tail is excluded. This indicates that different polyadenylation sites have been used by these sequences, an observation that is common in plant genes (Joshi 1987). Nucleotide sequences of both fragments, G2 and T1, have a 12-bp and 14-bp poly(A) tail, respectively. Their putative poly(A) signals are similar to ones described by Joshi (1987). The two bases immediately upstream of the poly(A) tail are CG in the case of G2, and AC in the case of T1, which are complementary to the anchored oligo dT primer (T12VG) and (T12VT), respectively. The 15-bp sequence at the 5′ ends of G1

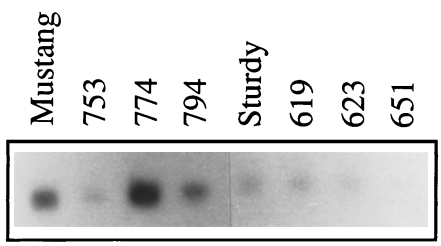

Fig. 4 Northern-blot hybridization of total RNA from heat-shocked seedlings of eight wheat lines [parental lines Mustang, Sturdy, six F_6 RILS including three thermotolerant lines (#753, #774, and #794) and three thermosusceptible lines (#619, #623, and #651)] using the differentially expressed 3′ cDNA fragment G2. Note the differential expression of an about 900-bp mRNA in thermotolerant lines and its absence in one thermotolerant and three thermosusceptible lines. Equal quantities (10 μg) of total RNA were loaded in each lane

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          1                                                   50
Tahsp266g2  CGCAAGGTCA TCGACGTGAA GGTCCAGTGA TGAGTACGTA TGTGCGAGAC
Tahsp266t1  CGCAAGGTCA TCGACGTGAA GGTCCAGTGA TGAGTACGTA TGTGCGAGAC
 Tahsp266a  CGCAAGGTCA TCGACGTGCA GGTCCAGTGA TGAGTTCG.. TGTGAGA..C
 Tahsp266b  CGCAAGGTCA TCGACGTGCA GGTCCAGTGA TGAGTTTG.. TGCGAGACTC

          51                                                  100
Tahsp266g2  TGTACCCTGT GTCTGAGGCT TTAAGATTTC AGCTGTCCCA ATATGACCGT
Tahsp266t1  TGTACCCTGT GTCTGAGGCT TTAAGATTTC AGCTGTCCCA ATATGACCGT
 Tahsp266a  TGTACCCTGC ATCTGAGGCT TTAAGATTTC AGCTGT.CCG AG........
 Tahsp266b  TGTACCCTGC ATCTGAGGCT TTGAGATTTC AGCTGT.CCG AGGCGTGACC

          101                                                 150
Tahsp266g2  CGGCGAGTGA GTGCTCTTTG TAGGCGTAGT GTGCCGTTCT TCTCCGATCC
Tahsp266t1  CGGCGAGTGA GTGCTCTTTG TAGGCGTAGT GTGCCGTTCT TCTCCGATCC
 Tahsp266a  ........GT GTGCTCTTTG TAG....AGT GTGCCGTTTT TCTCCAATCC
 Tahsp266b  GTGCGAGTGC GTGCTCTTTG TAGACGTAGG GTGCCGTTCT TCTCCGACCC

          151                                                 200
Tahsp266g2  TCTCTGCGCG CTCGTT.CGC AGAATACGTA GAGCTTTATG TAAATGTACT
Tahsp266t1  TCTCTGCGCG CTCGTT.CGC AGAATACGTA GAGCTTTATG TAAATGTACT
 Tahsp266a  TCTTTGCGCA CTCGTTGTGC GAAAGCGGCC GCGAATTC.. ..........
 Tahsp266b  TATCTGCGCG CTCGTTGCGC AGAACATGTC GTGCTGTACG TAAATGTACT

          201                                                 250
Tahsp266g2  TGGCAAATCA AT.GAATAAG TGTTCGGCTG CTTGATCAGC TGCACACACT
Tahsp266t1  TGGCAAATCA AT.GAATAAG TGTTCGGCTG CTTGCTCAGC TGGATACACT
 Tahsp266a  .......... .......... .......... .......... ..........
 Tahsp266b  TGGCAAATCA ATGGAATAAG TGTTCGGCTG GCGGCCGCGA ATTC......

          251                                 281
Tahsp266g2  TCGATTAATT ACTGCATCGA AAAAAAAAAA A
Tahsp266t1  TCGATTAACA AAAAAAAAAA AAA..... .
 Tahsp266a  .......... .......... .......... .
 Tahsp266b  .......... .......... .......... .
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Fig. 5 Nucleotide-sequence alignment of differentially expressed fragments 26.6 G2 and 26.6 T1, and of Tahsp26.6a and Tahsp26.6b (21, 25)

and T1 is identical to the HSP26.6 gene-family specific primer which had been used for fragment amplification. Overall the nucleotide sequences of G2 and T1 are highly homologous to the 3′ regions of HSP26.6a (77%) and HSP26.6b (81%) that were reported by us earlier (Weng et al. 1991; Nguyen et al. 1993). The first 27 base pairs from the 5′ end of G2 and T1 encode a polypeptide of nine amino acids which is identical at eight of nine amino acids with HSP26.6a and HSP26.6b. The third codon upstream from the stop codon encodes lysine in the fragments G2 and T1 instead of glutamine present in two other known members of this family in wheat. The isolation of the full-length cDNA identical to the G2 and T1 fragments is currently in progress.

Discussion

In this paper we have applied molecular and classical genetic approaches to the long-standing question of the significance of HSPs in acquired thermotolerance. Due to the complex nature of this phenomenon, it is unlikely that the thermotolerance trait can be accounted for by a single gene. A normal distribution curve for acquired thermotolerance, rather than a Mendelian segregation pattern, was observed in the F_2 progeny obtained from the cross between thermotolerant and thermosusceptible winter-wheat varieties 'Mustang' and 'Sturdy,' thus confirming that this trait is not regulated by a single gene (Hendershot and Nguyen, unpublished

observation). Acquired thermotolerance is probably a multigene trait with several components, each of which accounts for a certain portion of the genetic variation. Thus, every evidence suggesting the significance of a genetic component in acquired thermotolerance is invaluable, even if the expression of this gene results in only a small increase of the plant's acquired thermotolerance capacity. To-date, limited information exists regarding the genetic association between the expression of a specific HSP and thermotolerance in plants, although in many cases induced expression of HSPs was correlated with the development of the thermotolerant state (O'Connel 1994). While positive associations were found between genotypic differences in heat tolerance and HSP production in sorghum (Howarth 1989; Ougham and Stoddart 1986), sugarcane (Moisyadi and Harrington 1989), wheat (Krishnan et al. 1989), and maize (Jorgensen et al. 1992), it has never been determined whether any specific HSP is genetically linked to heat tolerance in these plants. Recently, Park et al. (1996) demonstrated a genetic linkage between extra HSP25 polypeptides and heat tolerance in creeping bentgrass.

Here we report that the expression of a specific member of the HSP26 family co-segregates with the acquired thermotolerance trait as determined by a TTC cell-viability assay. Due to the limited number of RILs that we tested, these data need to be further validated. More RILs of a further advanced generation (such as the F_8) with a higher homozygosity level will be further tested to conclusively prove this association.

Investigations in our laboratory have found that the TTC cell-viability assay is an effective screening tool for whole-plant thermotolerance and that this trait is highly heritable (Krishnan et al. 1989; Vierling and Nguyen 1992; Porter et al. 1994, 1995). The cell viability measured by the TTC assay was correlated with chlorophyll stability in flag leaves of 'Mustang' and 'Sturdy' under chronic heat stress (Nguyen et al. 1994) as well as with grain filling and grain yield of wheat under heat stress (Fokar 1994).

We have identified a novel isoform of HSP26 (HSP26-II) that was synthesized under heat-stress conditions in the thermotolerant cultivar 'Mustang' and the thermotolerant F_6 RILs but was absent from the thermosusceptible cultivar 'Sturdy' and thermosusceptible F_6 progeny. Two polypeptides of an approximate molecular weight of 22 kDa hybridized with polyclonal anti-wheat HSP26 antibodies on 2-D Western blots in 'Mustang,' while only one 22-kDa polypeptide was observed in 'Sturdy.' The differences in HSP26 accumulation between 'Mustang' and 'Sturdy' are likely to be due to the expression of this additional member of the HSP26 family (HSP26-II) in 'Mustang.'

We have isolated specific cDNA probes related to a unique member of the HSP26 gene family using the modified differential display method with a HSP26 gene-family specific primer. The G2/T1 cDNA can be considered a 'gene-specific' probe, because it specifically recognizes the 'Mustang'-specific mRNA homologous to the HSP26 gene family. Our preliminary data using the G2/T1 probe also indicates differential expression of a HSP26 family member in thermotolerant and thermosusceptible varieties of spring wheat that are genetically distant from winter wheat (Klueva and Nguyen, unpublished observations). This observation suggests that this phenomenon may be of general significance for wheat as a species.

Coordinated expression of an mRNA corresponding to the G2/T1 cDNA fragment and HSP26-II polypeptide in all tested RILs (with one exception) suggests that these events may be related. The only exception is line #753 in which we observed expression of the HSP26-II isoform at the protein level and absence of mRNAs homologous to G2/T1. This RIL was the least thermotolerant of all thermotolerant lines (Table 1) and its HSP26-II polypeptide expression was less pronounced than in the three other thermotolerant lines (Fig. 2).

In the current study, we obtained a protein marker and a cDNA probe, both related to the HSP26 gene family, which are closely associated with acquired thermotolerance in winter wheat. Sequencing of the HSP26-II polypeptide, as well as obtaining and sequencing of the full-length cDNA corresponding to G2/T1, is necessary to elucidate the relationship between them. We hypothesize that the 'Mustang'-specific HSP26 polypeptide (HSP26-II) is independently translated from the HSP26 gene present and is heat-stress-induced only in 'Mustang' and 'Mustang'-like RILs while absent or inactive during heat stress in 'Sturdy'-like thermosusceptible lines. A high percentage of homology between G2/T1 and previously cloned members of the HSP26 family from wheat HSP26a and HSP26b (Weng et al. 1991; Nguyen et al. 1993) (Fig. 5) suggests that these sequences belong to the same class of HSP26 members. Both HSP26a and HSP26b genes have been isolated from the thermotolerant cultivar 'Mustang' (Weng et al. 1991; Nguyen et al. 1993) Hence, other member(s) of the HSP26 gene family present in both 'Mustang' and 'Sturdy', whose heat-inducible expression results in the HSP26-I polypeptide observed in both cultivars, need to be isolated to confirm this hypothesis.

Another possibility is that the presence of the HSP26-II polypeptide in the 2-D patterns of total protein fractions in 'Mustang' and thermotolerant RILs results not from an independent gene but from a post-translational modification of the HSP26-I polypeptide, leading to a shift of the isoelectric point from pl 5.5 (observed for HSP26-I) to pl 5.0 (observed for HSP26-II) in these lines (Fig. 2). Phosphorylation is one of the likely modifications that may result in a pl shift and this modification by itself may be a significant factor for thermotolerance. In this case, G2/T1-homologous mRNA would not be directly related to the expression of HSP-II.

What is the likely function of the HSP26-II polypeptide in protecting plant cells during heat stress? The HSP26 gene family contains plastid-localized HSPs that are known to be post-translationally transported to plastids (Vierling 1991) where they are found in the stroma, indicating that they may protect photosynthetic enzymes from heat damage. The HSP26-II polypeptide may be performing a similar protective function inside chloroplasts. More research needs to be conducted into the structure and function of this protein during heat stress. The results could decipher the function of different HSP26 members in the acquired thermotolerance of crops. To further understand the function of HSP26, we are currently performing reverse-genetics experiments, expressing antisense HSP26 constructs in transgenic plants.

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