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RESEARCH PAPER

Screening of inbred lines to develop a thermotolerant sunflower hybrid using the temperature induction response (TIR) technique: a novel approach by exploiting residual variability

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Abstract

Plants, when exposed to sub-lethal stress (induction stress), develop the ability to withstand severe temperatures and this phenomenon is often referred to as acquired thermotolerance. Earlier it was reported that induction stress alters gene expression and brings greater adaptation to heat stress and that the genetic variability in thermotolerance is only seen upon induction stress. Based on this concept, the temperature induction response (TIR) technique has been developed to identify thermotolerant lines. By following the TIR technique, sunflower hybrid KBSH-1 parents were screened for high temperature tolerance. Seedlings of parental lines including CMS 234 A, CMS 234 B and 6 D-1 showed considerable genetic variability for thermotolerance and it was attributed to the expression of existing residual variability for stress responses. Thus, the existing variability forms the basis for identifying thermotolerant lines. The identified parental inbred lines were selected and established in the field and crossed to get F_1 hybrid seeds. The KBSH-1 hybrid developed from selected variants of parental lines was compared with the original KBSH-1 for thermotolerance. The selected KBSH-1 was more tolerant compared with the original hybrid both at the seedling as well as at the plant level. The physiological and molecular basis of thermotolerance was studied in the KBSH-1 original and the hybrid developed from selected variants of parental lines. The selected hybrid exhibited high tolerance to Menadione (naphthoquinone)-induced oxidative stress. Even the methyl viologen-induced oxidative stress damage was relatively less in the selected hybrid population. The selected hybrid also showed enhanced expression of the heat shock proteins HSP 90 and HSP 104 and also accumulated higher levels of the heat shock transcription factor HSFA.

Key words: *Helianthus annuus* L, heat shock protein, heat stress, residual variability, temperature induction response.

Introduction

In the tropics the mean high temperatures during the day and night substantially decreases the growth rates and productivity of many crop species, especially that of temperature-sensitive crops like sunflower. Despite the high intrinsic yield potential of F_1 hybrids under optimum conditions of growth, the yields often realized under high temperatures are relatively low. Apart from effects on the duration of growth stages, especially the grain-filling period, high temperature has a deleterious effect on several physiological processes (Stone and Nicolas, 1984). Heat stress causes substantial membrane damage and leaf senescence (Liu and Huang, 2000). It has been believed that, in addition to the avoidance mechanisms such as

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excess heat dissipation through evaporative cooling, intrinsic tolerance mechanisms are more relevant for a greater adaptation to high temperature.

To cope up with the changing environmental conditions, plants synthesize a set of specific stress-responsive proteins and they are involved in altering specific biochemical processes necessary for adaptation (Neumann *et al*., 1989; Holmstrom *et al*., 1994; Hayashi *et al*., 1997; Shen *et al*., 1997; Deak *et al*., 1999). One of the most widely studied aspects of thermotolerance is the enhanced expression of HSPs. Synthesis and localization of a few HSPs have been shown to trigger several physiological and biochemical processes (Chen *et al*., 1990; Cushman and Bohnert, 2000) such as the maintenance of membrane integrity (Berry and Bjorkman, 1980; Kader *et al*., 1991) and chaperoning proteins (Sanchez and Lindquist, 1990; Vierling and Nguyen, 1992). The expression of HSPs is primarily regulated by the heat-dependent activation of the heat shock transcription factors (HSFs) (Scharf *et al*., 1998) Further, the functional relevance of HSPs has been convincingly demonstrated by their overexpression in transgenic plants (Malik *et al*., 1999), by down-regulation through an antisense approach (Lee and Schoffl, 1996), and through knockout studies (Burke *et al*., 2000; Hong and Vierling, 2000). Correlative studies such as the induction response (Park *et al*., 1996; Yong *et al*., 1997) and genetic variability studies (Krishnan *et al*., 1989; Moons *et al*., 1995; Jayaprakash *et al*., 1998; Kumar *et al*., 1999) indirectly explain the role of HSPs in thermotolerance.

Genetic variability in the stress response has been suggested to be mainly due to the differential expression of stress-responsive genes (Krishnan *et al*., 1989; Fender and O'Connell, 1990; Joshi *et al*., 1997). There is convincing evidence to show that the stress-responsive genes are predominantly expressed during the sub-lethal induction stress that would bring the required changes in the plant metabolism necessary for withstanding the subsequent severe stress (Lindquist and Craig, 1988). Several studies (Uma *et al*., 1995; Jayaprakash *et al*., 1998; Kumar *et al*., 1999; Burke, 2001; Srikanthbabu *et al*., 2002) have clearly shown that genetic variability for the stress response could only be seen upon exposure to an induction stress. Therefore, to assess stress tolerance, it is necessary to expose the plants to an induction stress before exposing them to the severe stress. Based on preliminary studies, an efficient screening technique referred to as the temperature induction response (TIR) technique has been developed to identify thermotolerant lines. According to this technique, the seedlings are exposed to an optimum induction temperature before being exposed to a severe challenging temperature and subsequently allowed to recover at room temperature. The surviving seedlings at the end of the recovery period are selected as thermotolerant lines (Kumar *et al*., 1999; Srikanthbabu *et al*., 2002). Earlier studies clearly show that TIR is an effective technique for screening for high temperature tolerance (Kumar *et al*., 1999; Srikanthbabu *et al*., 2002) and, by following this technique, thermotolerant lines were identified from a sunflower population, cv. Morden, which showed better performance than the original population under high temperature (Kumar *et al*., 1999; Mamatha Reddy, 2000).

It is hypothesized that induction stress, which is a prerequisite for the optimum expression of stress-responsive genes, brings about intrinsic differences in stress tolerance amongst the different lines within a population, which is otherwise uniform for several agronomic traits. It is reported here that considerable variability exists, not only in a sunflower varietal population but also in inbred lines, for thermotolerance. The F_1 hybrid developed from selected thermotolerant variants of male and female parental inbred lines showed a higher degree of temperature tolerance. Further, this thermotolerant hybrid showed enhanced expression of HSPs and also heat shock transcription factors like HSFA.

Materials and methods

Plant material

The sunflower (*Helianthus annuus* L.) parental line (inbreds) seeds of KBSH-1 hybrid, CMS 234 A (female parent), CMS 234 B (maintainer line), 6 D-1 (restorer line), and cv. Morden (an open-pollinated heterogeneous population) were procured from National Seeds Project (NSP), UAS, Bangalore, India. About 2-d-old seedlings germinated on filter paper in Petri dishes and 30-d-old plants raised in battery pots (25 kg soil) were used for the experiments.

Challenging temperature

Seedlings of sunflower cv. Morden were subjected to different temperatures (49, 50, 51, and 52 °C) for 1, 2 and 3 h without prior induction and these seedlings were immediately allowed to recover at 30 °C for 72 h in an incubator. At the end of the recovery period the temperature treatment at which 90% mortality of the seedlings occurred was taken as the challenging temperature in order to assess the genetic variability for seedling survival. The temperature treatment at which 50% reduction in seedling growth occurred was considered as the challenging temperature to assess differences in recovery growth.

Determination of optimum induction treatments for seedlings

Seedlings of sunflower cv. Morden (2-d-old) were subjected to different induction temperature treatments following which they were transferred to a defined challenging temperature. (1) Temperature was increased from 28 °C to 42 °C in 2.5 h and maintained at 42 °C for 2 h; (2) temperature was increased from 28 °C to 44 °C in 4.5 h; (3) temperature was increased from 28 °C to 45 °C in 5 h; and (4) seedlings were maintained at 35 °C for 1 h, 40 °C for 1 h and 45 °C for 2 h and immediately transferred to the challenging temperature.

Recovery growth

After subjecting the seedlings to challenging temperature, seedlings were allowed to recover at 30 °C for 72 h. At the end of recovery, the percentage survival and root and shoot growth of the surviving seedlings were recorded. The root and shoot length (cm) was

measured to arrive at total seedling growth. In all the experiments two replications were maintained per treatment, and each replication had 25 seedlings.

The seedlings and/or plants which are maintained at 30 °C (normal temperature) throughout the experimental period were taken as absolute controls.

The percentage reduction in growth was calculated by dividing the difference of the growth of the control and the treatment by the growth of the control.

The induction temperature treatment at which maximum percentage survival of the seedlings was observed after exposing to the defined challenging temperature (51 °C for 2 h) was considered as the optimum induction temperature (Fig. 1).

The coefficient of variation for the recovery growth of seedlings was calculated using the formulae

$$\text{Coefficient of variation} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Temperature stress response of KBSH-1 hybrid parental lines

The seedlings of parental lines (CMS 234 A, CMS 234 B and 6 D-1) were subjected to different temperature treatments to arrive at the optimum challenging temperature for 90% seedling survival and 50% reduction in recovery growth. Subsequently, the optimum induction treatment was calculated for these parental lines by subjecting the seedlings to different induction treatments.

The extent of variation in seedling recovery growth within a population was examined by exposing seedlings to challenging temperature (49 °C for 2 h) following an optimum induction.

Identification of thermotolerant parental lines

The primary objective of the study was to identify highly tolerant plants from each parental line (CMS 234 A, CMS 234 B and 6 D-1). In view of this the challenging temperature was altered in such a way to ensure that only 30% seedlings survived following the optimum induction treatment. This temperature was selected as the high stringency temperature stress and used for screening the parental lines. By adopting the high stringency challenging temperature protocol about 30 000 seeds of each parental lines were screened and the surviving seedlings were planted in the field. Only about 60% of the selected seedlings were established as plants in the field. Subsequently, only the phenotypically uniform plants were maintained for crossing. After screening, subsequent establishment in the field and culling, approximately 2% of the total seeds taken for screening were developed as a thermotolerant population in each parental line. These parental lines were designated as selected variants.

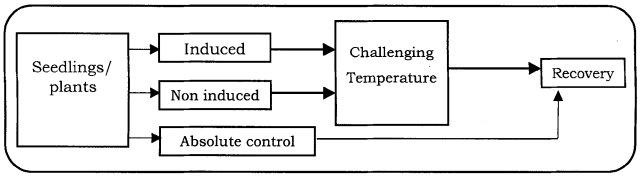

Fig. 1. The general protocol followed in order to study the induction response of sunflower seedlings and plants. Induced: seedlings or plants subjected to an induction temperature treatment and immediately transferred to the challenging temperature. Non-induced: temperature maintained at 30 °C then exposed to the challenge temperature. Absolute control: temperature maintained at 30 °C throughout the experiment. Challenge temperature: 49 °C for 2 h and 51 °C for 2 h. Recovery: temperature maintained at 30 °C for 72 h.

Crossing and seed production

The F_1 hybrids were obtained by hand pollination of CMS 234 A with 6 D-1. The F_1 hybrids developed from the selected thermotolerant parental lines were referred to as the selected KBSH-1 hybrids whereas the F_1 hybrids developed from the original non-selected parental lines were referred to as the non-selected KBSH-1 hybrids. The selected variants of the CMS 234 A line were crossed with selected variants of the CMS 234 B line to obtain the subsequent progeny of CMS 234 A. Similarly, the selected variants of 6 D-1 and CMS 234 B were intercrossed to obtain the subsequent generation of 6 D-1 and CMS 234 B, respectively.

RAPD analysis

To study the genetic variability amongst the seedling population within an inbred, RAPD analysis was carried out in seedlings differing in stress recovery growth.

DNA was extracted from seedlings according to the methods of Saghai-Maroof *et al*. (1984) with slight modifications. RAPD amplification was performed in a reaction volume of 25 µl containing 1× TRIS-HCl (pH 8), 2 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of dNTPs, 5 pmol primer, 50–75 ng genomic DNA, and 1 unit *Taq* DNA polymerase. Amplifications were performed on a PTX-100 thermocycler (MJ Research Inc., Wahrtown, MA, USA) at 94 °C for 2 min, 35 cycles of 2 s at 94 °C, 2 s at 35 °C, 90 s at 72 °C followed by an 8 min extension at 72 °C.

Primers (10 mers) were purchased from Metabion GMbH, Lena-Christ-Strasse 44, Deutschland, were used for the study.

Stress tolerance of selected parental lines

Thermotolerant parental lines established in field were compared for oxidative stress tolerance with the original parental lines. To study the oxidative stress tolerance, the excised leaf punches collected from the top third leaf of 30-d-old plants were incubated in 2 µM methyl viologen for 4 h in dark at 30 °C and subsequently exposed to a temperature of 40 °C or 45 °C with 1000 µmol m^{-2} s^{-1} light intensity for 2 h using a temperature- and humidity-controlled chamber with an artificial light source. The RH in the chamber was maintained at 90%. The membrane integrity in the leaf punches was analysed 5 h after the oxidative stress treatment by following the method of Leopold *et al*. (1981).

Stress response of selected KBSH-1 hybrid

Thermotolerance: Seedlings of both the selected hybrid and the original hybrid were challenged with different temperatures following an optimum induction temperature treatment. Later, the seedlings were allowed to recover at 30 °C with 60% relative humidity for 72 h in the incubator and, at the end of recovery period, growth was recorded.

Oxidative stress tolerance: Oxidative stress tolerance was assessed using a redox cyclic compound Menadione (2-methyl-1,4-naphthoquinone) (Reichheld *et al*., 1999). Seedlings were incubated in 2 mM Menadione for 3 h. Later, the seedlings were allowed to recover in water at 30 °C with 60% relative humidity for 72 h in the incubator and, at the end of recovery period, growth was recorded and expressed as the percentage reduction over absolute control. In a subset of seedlings the percentage membrane damage was assessed 5 h after the Menadione treatment using a non-toxic water-soluble dye, Evans Blue (Turner and Novacky, 1974). Membrane damage was assessed by incubating the Menadione-exposed seedlings to 0.05%

of Evans Blue for 2 h. The dye bound to damaged cells was solubilized in 100% methanol for 30 min at 50 °C and quantified by absorbance at 600 nm using spectronic genesys-2.

Plant level

The 30-d-old selected KBSH-1 hybrid parents were challenged with 51 °C for 1 h with and without prior induction. Later, they were allowed to recover in the greenhouse for 6 d. Leaf area developed during the recovery period was recorded. Oxidative stress tolerance was also measured using methyl viologen as described earlier.

Western blot

Proteins, isolated from sunflower seedlings and leaf tissues in 0.1 M TRIS-HCl buffer (pH 7.8) containing the protenase inhibitor, PMSF, and also benzamidine, were subjected to SDS-PAGE 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 the rice primary antibodies HSP 90, HSP 104 and HSFA raised in rabbit. The bands in the western blot were visualized after incubating with alkaline phosphatase-conjugated IgG (1:1000 dilution) for 1 h at room temperature and developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as a substrate (Engvall and Perlmann, 1972).

Preparation of HSFA antibodies

A conserved peptide of 21 amino acid sequence present in the HR A/B of HSFA was identified using a multiple sequence alignment programme. Based on the results, a 16-mer sequence of YQQQQQSTDNQLQNQK was deduced. Peptide was synthesized for the conserved sequence and was conjugated to the carrier protein BSA using the glutaraldehyde single step coupling method (Harlow and Lane, 1988). By immunizing New Zealand rabbits with this peptide, the polyclonal antibody was developed.

Results

Optimum induction temperature for sunflower cv. Morden

Seedlings of cv. Morden were exposed to different challenging temperatures and, after recovery, seedling growth and seedling mortality was assessed (Fig. 1). The optimum induction temperature was arrived at by challenging the seedlings to a defined challenging temperature following different induction treatments. Seedlings exposed to the gradual induction temperature (temperature raised from 28 °C to 42 °C in 2.5 h and maintained at 42 °C for 2 h) prior to the challenging temperature exhibited higher seedling survival and recovery growth compared to those exposed to other induction treatments and the non-induced seedlings (Fig. 2) At 49 °C for 2 h, 90% reduction in recovery growth was observed whereas at 51 °C for 2 h, 90% seedling mortality was seen in non-induced seedlings. These results indicated that the induction response was seen both in recovery growth and also in seedling survival. Since cv. Morden is a population, as expected significant variability was seen in the seedling population both in survival and recovery growth. Variation in the recovery growth amongst the population was only seen in the induced seedlings exposed to stress.

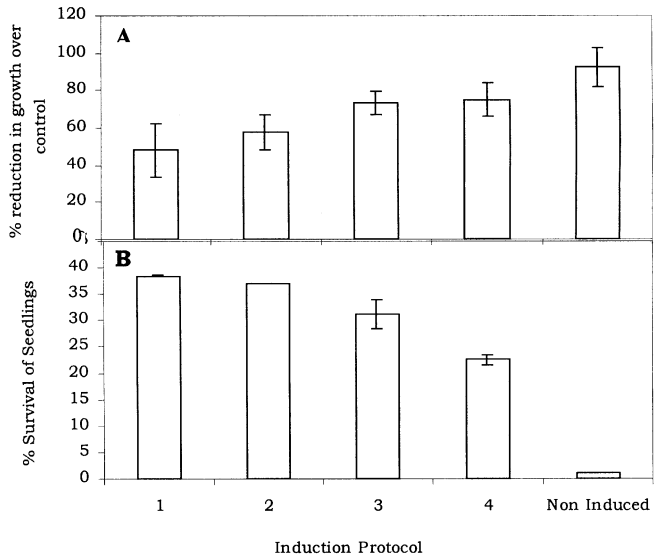

Fig. 2. The induction response of sunflower seedlings cv. Morden to temperature stress. Seedlings of sunflower cv. Morden were exposed to different induction treatments separately and then subsequently exposed to either 49 °C for 2 h (A) or 51 °C for 2 h (B). (1) Temperature was increased from 28 °C to 42 °C in 2.5 h and maintained at 42 °C for 2 h. (2) Temperature was increased from 28 °C to 44 °C in 4.5 h. (3) Temperature was increased from 28 °C to 45 °C in 5 h. (4) Seedlings were maintained at 35 °C for 1 h, 40 °C for 1 h and 45 °C for 2 h and immediately transferred to the challenging temperature. The seedlings were allowed to recover at 30 °C for 72 h. At the end of recovery the percentage survival and recovery growth were determined. Three independent experiments were conducted and the data were averaged. Bars represent the standard error of means at the 5% level.

Table 1. *Coefficient of variation values (percentage) for recovery growth of seedlings within inbred lines and Morden population after exposing to temperature stress*

Seedlings were challenged with 49 °C for 2 h following optimum induction temperature treatment, immediately the seedlings were allowed to recover at a temperature of 30 °C for 72 h. At the end of recovery root and shoot growth (cm) was recorded and deviation in growth from the total arithmetic mean were determined and expressed as coefficient of variation values. In each treatment about 200 seedlings were assessed.

	CMS 234 A	CMS 234 B	6 D-1	Morden
Absolute control	19.9	16.2	13.5	46.25
Stressed	55.2	51.25	42.0	85

The differences in seedling growth were marginal under non-stress conditions (Table 1). To examine whether such variability is expressed under stress even in inbred populations, the inbred lines of KBSH-1 hybrid were screened.

Assessment of genetic variability in KBSH-1 parental line seedlings

In all the three parental lines of the KBSH-1 hybrid (CMS 234 A, CMS 234 B, 6 D-1) a gradual induction temperature treatment (28 °C to 42 °C in 2.5 h and 42 °C maintained for 2 h) was found to be optimum.

By adopting this protocol, the induction response was examined by exposing the seedlings to 49 °C for 2 h. In all three parental lines recovery growth was markedly high upon induction (1–2-fold). Interestingly, a large variation in recovery growth (root and shoot length) of induced seedlings was seen with in the inbred population. However, such a large variation was not seen in non-stressed seedlings. The coefficient of variation values given in Table 1 clearly indicates that significant variability in recovery growth was seen within the inbred population on exposure to temperature stress.

RAPD analysis of resistant and susceptible seedlings within an inbred population

Based on the differences in recovery growth of induced seedlings after exposure to a severe stress, seedlings were classified into susceptible and resistant populations. To assess whether these two groups differ genetically, the polymorphism between these susceptible and resistant seedlings was studied by RAPD analysis using several random primers. Amongst 25 primers screened three of them (OPM20, OPO16 and OPO17) showed polymorphism between these two groups of seedlings both in CMS 234 A and 6 D-1 (Fig. 3).

Selection of thermotolerant lines from the inbred parental lines of sunflower hybrid

Since considerable variation was seen for temperature tolerance in the inbred population of each parental line, a screening protocol was developed to identify highly tolerant lines in each parent. The objective was to arrive at a challenging temperature where only 30% of induced seedlings survive on recovery. This temperature was considered as the high stringency challenging temperature. Interestingly, significant variation was seen among parental lines for thermotolerance. CMS 234 A, that is relatively more thermotolerant, showed 70% seedling mortality at the challenging temperature of 52 °C for 2 h, whereas the susceptible parental line, 6 D-1, showed about 70% seedling mortality at 50 °C for 2 h. Since the threshold temperature for the survival of 30% seedlings was different for these inbred lines subsequent screening was done at 52 °C for 2 h for CMS 234 A, at 51 °C for 2 h for CMS 234 B and at 50 °C for 2 h for 6 D-1.

Approximately 30 000 seeds of each parental line were screened (Fig. 4). The seedlings survived after high stringency temperature treatment was established in the field. The crossing was done between phenotypically uniform plants and F_1 hybrid seeds were obtained. Simultaneously, the plants developed from original parental lines were also crossed to get F_1 hybrids.

Assessment of oxidative stress tolerance of parental lines at plant level

The plants established in the field from screened seedlings were tested for oxidative stress tolerance. Leaf punches collected from 40-d-old plants of selected and original parental lines were subjected to oxidative stress. The level of stress tolerance was assessed by determining the membrane integrity. The selected variants of parental

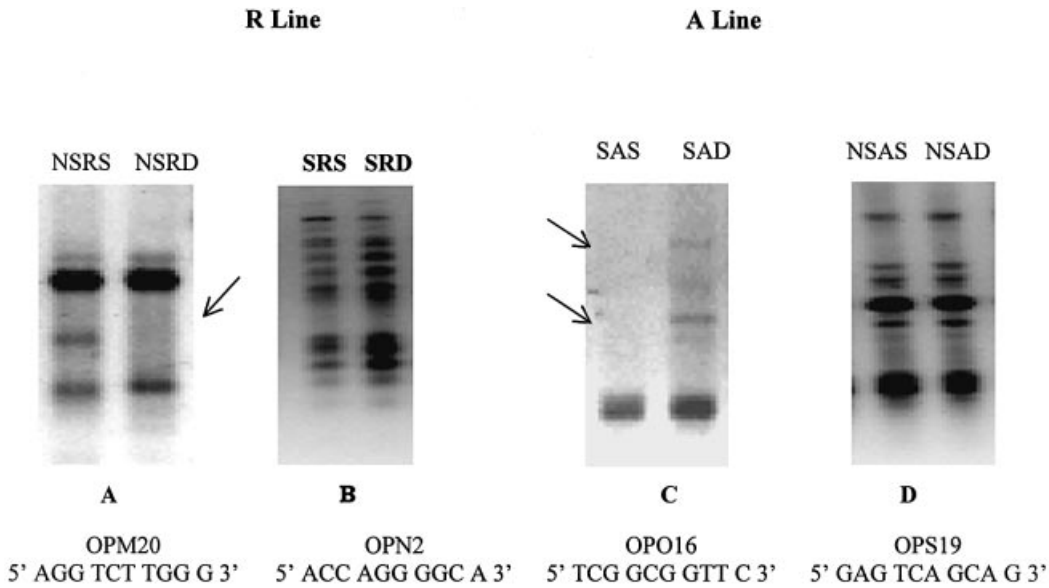

Fig. 3. RAPD profile showing the polymorphism in susceptible and resistant seedlings of CMS 234 A and 6 D-1 lines. Contrasting seedlings differing in recovery growth were screened with RAPD primers. The primers OPM20 and OPO16 showed distinct polymorphism (A, C) where as OPN2 and OPS19 showed a similar banding pattern. NSRS, non-screened R line surviving; NSRD; non-screened R line dying; SRS, screened R line surviving; SRD, screened R line dying; SAS, screened A line surviving; SAD, screened A line dying; NSAS, non-screened A line surviving; NSAD, non-screened A line dying.

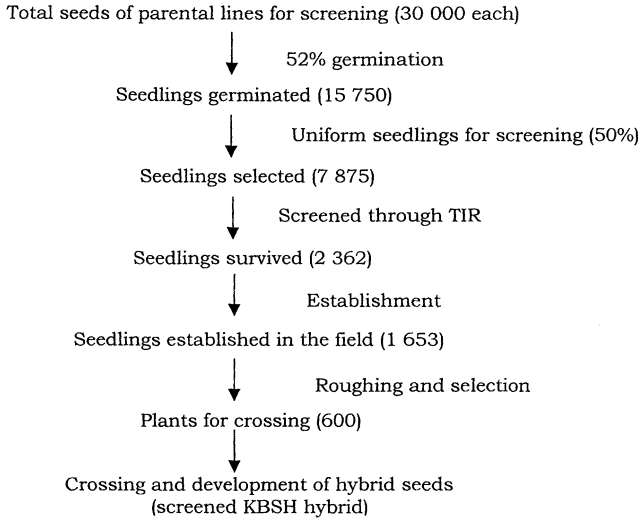

Fig. 4. Screening of parental lines and development of the thermotolerant KBSH-1 hybrid. The details given are for CMS 234 A, but a similar trend was seen with other lines.

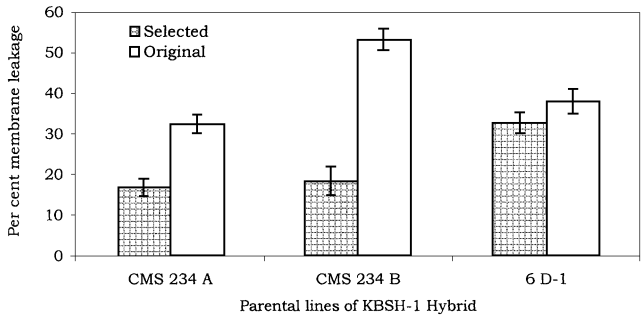

Fig. 5. Higher membrane stability in the selected parental lines under high temperature coupled with photoinhibitory conditions. Leaf punches were incubated in 2 μM methyl viologen for 4 h and exposed to 1000 μmol m^{-2} s^{-1} of light at a temperature of 45 °C for 2 h. After 5 h of recovery, membrane leakage was measured (Leopold *et al*., 1981). Three independent experiments were conducted and the data were averaged. Bars represent the standard error of means at the 5% level.

lines (CMS 234 A and CMS 234 B) exhibited higher membrane integrity compared to original plants (Fig. 5).

Stress tolerance of the hybrids developed from selected and original parental lines

Seedling level: Relative thermotolerance and oxidative stress tolerance of selected and original hybrids were assessed. The selected hybrid seedlings showed substantially higher thermotolerance. The recovery growth was markedly high at different lethal temperature in selected hybrid except at 51 °C for 3 h (Fig. 6).

Even under the oxidative stress condition induced by treating seedlings with Menadione the recovery growth was high in the selected hybrid seedlings and they also maintained relatively high membrane integrity (Figs 7, 8A).

Plant level: Thermotolerance of the pot-grown 30-d-old plants of selected and original hybrids was assessed by monitoring recovery growth after subjecting to high temperature stress. Methyl viologen-induced oxidative stress tolerance was also assessed in excised leaves by quantifying the extent of membrane integrity. The selected hybrid exhibited a higher recovery growth (Fig. 8B) and membrane integrity under stress (data not shown).

These results confirm that the selected hybrids developed from the selected variants are intrinsically tolerant to both high temperature and oxidative stress.

Expression of HSPs and HSFs in hybrids

One of the possible mechanisms for the enhanced survival and recovery growth of selected hybrid seedlings is by the synthesis of heat shock proteins during induction stress. Therefore, the expression pattern of known stress-responsive genes HSP 90, HSP 104 and also the heat shock transcription factor HSF was examined at the end of induction in the selected and original hybrids.

Antibodies developed for a conserved amino acid stretch specific to HSFA between heptad repeats of the trimerization domain were used to determine the level of HSF in the selected and original hybrid plants. The HSF (55 kDa) showed substantially higher levels of expression upon induction in selected hybrids compared to the original hybrid (Fig. 9A).

In the seedlings of selected hybrids, the level of expression of HSP 90 was high at the end of induction treatment (Fig. 9B). Even at the plant level the high molecular weight HSP 104 showed relatively higher expression in hybrid plants developed from thermotolerant selected variants of parents (Fig. 9C).

Discussion

Sub-lethal induction stress which the plants experience under natural conditions before being subjected to severe stress, triggers the expression of an array of stress-responsive genes and these gene products alter several physiological and biochemical processes relevant to stress tolerance (Vierling, 1991; Bohnert *et al*., 1995). It has been observed that genetic variability is only seen upon an induction treatment prior to severe stress (Krishnan *et al*., 1989; Uma *et al*., 1995; Jayaprakash *et al*., 1998; Kumar *et al*., 1999; Srikanthbabu *et al*., 2002) and the observed variability was marginal when the seedlings were exposed directly to the severe stress (Uma *et al*., 1995). Therefore, while screening for thermotolerance, it is necessary to expose the seedlings to an induction stress before its exposure to the severe stress.

By adapting the temperature induction response (TIR) technique, the existence of significant genetic variability

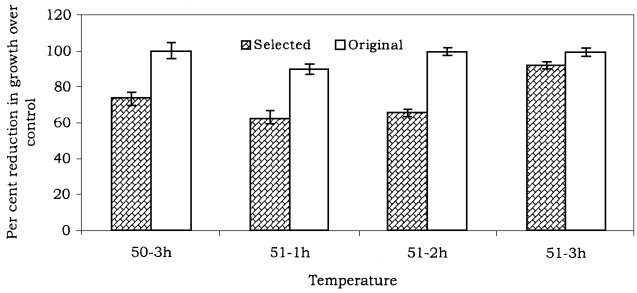

Fig 6. Differences in recovery growth of the seedlings of selected and original KBSH-1 hybrids. Seedlings of both selected and original hybrids were challenged with 50 °C for 3 h, 51 °C for 1 h, 51 °C for 2 h, 51 °C for 3 h following an optimum temperature induction. The recovery growth was recorded after the recovery period of 72 h at 30 °C and 60% RH. The percentage reduction in growth over absolute control was calculated. In all the treatments non-induced seedlings did not survive. Three independent experiments were conducted and the data were averaged. Bars represent the standard error of means at the 5% level.

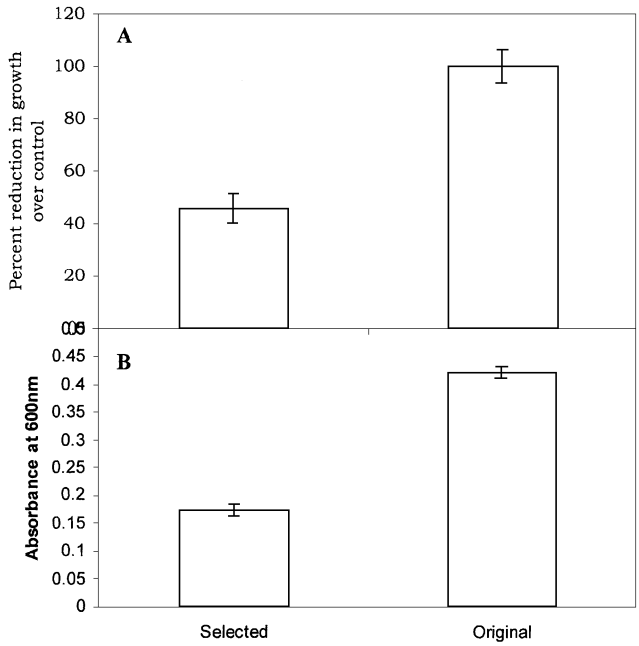

Fig 7. Oxidative stress tolerance of selected KBSH-1 hybrid seedlings. Seedlings were subjected to 2 mM Menadione for 3 h in the dark and then allowed to recover at 30 °C with 60% relative humidity for 72 h, before recovery growth, in terms of the reduction in growth over the control (A), was measured. From a subset of seedlings after 5 h of recovery the membrane integrity was assessed using Evans Blue (for details see Materials and methods) (B) and the absorbance values expressed on unit tissue fresh weight basis (0.5 g).

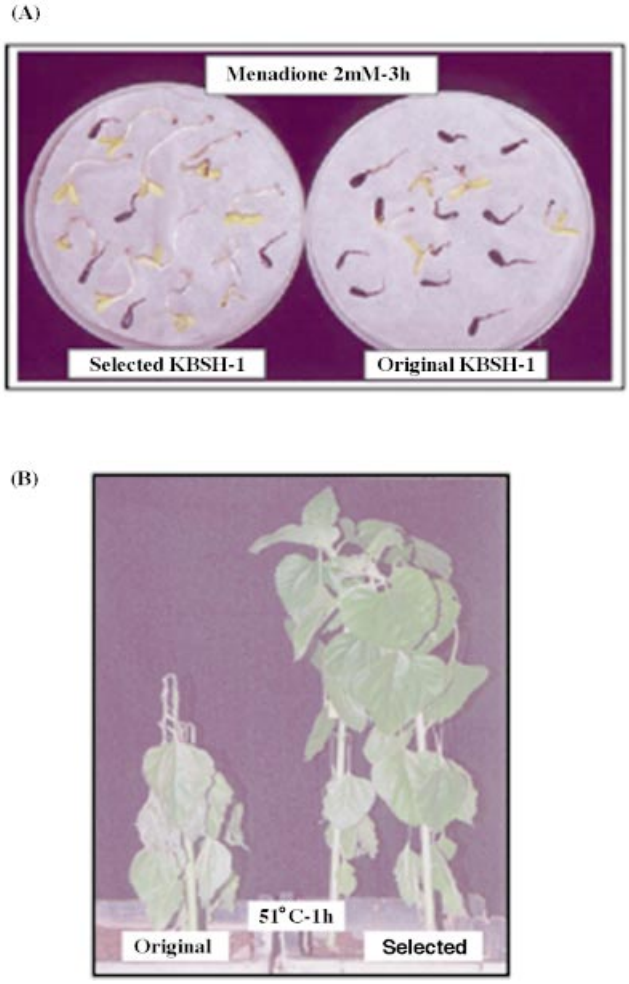

Fig. 8. (A) Variation in the oxidative stress tolerance of selected and original KBSH-1 hybrids at the seedling level. The seedlings were challenged with 2 mM Menadione for 3 h and allowed to recover. The photograph was taken after 72 h of recovery. (B) Variation in the thermotolerance of selected and original KBSH-1 hybrids at the plant level. The plants (30-d-old) developed from selected and original parental lines were subjected to a challenging temperature of 51 °C for 1 h following optimum temperature induction. The photograph was taken 6 d after recovery.

has been demonstrated across the genotypes of pea, sunflower and groundnut and thermotolerant lines from the sunflower open-pollinated population, cv. Morden (Kumar *et al*., 1999; Mamatha Reddy, 2000; Srikanthbabu *et al*., 2002) have also been identified. The sunflower cv. Morden is an open-pollinated variety and it is homozygous for many agronomic traits like phenology, time to flowering and head characters etc. However, considerable variation for thermotolerance was seen within the population. The results of the present investigation show least difference in the growth rate amongst the seedlings under non-stress conditions in the Morden population. The coefficient of variation for seedling recovery growth of non-stressed seedlings from open-pollinated populations (Morden) was only 46.25%. However, when the seedlings were exposed to an optimum induction treatment before high temperature stress, the observed variation was about 85% indicating that considerable variability for thermotolerance exists, even though the population is homozygous for various other traits.

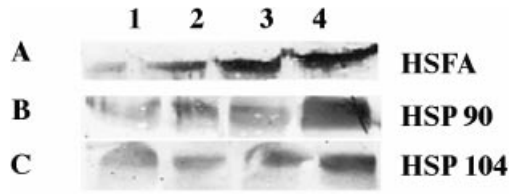

Fig. 9. Western blot showing the expression of HSFA (A), HSP 90 (B) and HSP 104 (C) in selected and original hybrids. Total soluble protein was extracted from induced and non-induced seedlings (A, B) and leaves (C) of selected and original KBSH-1 hybrids. 100 μg of protein was resolved on 10% SDS-PAGE and later transferred to a nitrocellulose membrane and immunoblotted and probed with polyclonal antibodies of HSFA, HSP 90 and HSP 104 (1:1000 dilution). Lane 1, original KBSH-1 control (non-induced); lane 2, original KBSH-1 (induced); lane 3, selected KBSH-1 control (non-induced); lane 4, selected KBSH-1 (induced).

To assess whether residual variability exists in inbred lines for stress adaptive traits, optimum induction protocols were developed for all the three inbred lines and the extent of variability was examined. Variation in seedling growth within an inbred population when grown under control conditions was minimal, but the variation in the recovery growth on exposure to stress was significant (Table 1). Therefore, within inbred lines there is variation for the traits for which the selection was not made during the process of inbred development. Distinct RAPD polymorphism was seen between the resistant and susceptible groups within an inbred, which confirms the existence of genetic variability (Fig. 3). This variation could be attributed to residual variability present in the inbred population. There are reports that show the existence of variation in the parental lines of hybrids of *Coffea arabica* for yield, stem diameter and height (Cilas *et al*., 1998) and in tomato and potato (Tanksley, 1992). Guillen-Portal *et al*. (1999) showed that, under the single plant selection scheme, even though the grain amaranth reached near homozygosity in the F_5 generation, analysis of the F_5 generation showed residual variability and it may be present indefinitely. The possible causes for residual variability have been suggested to be due to homologous pairing, new mutations and recombination of linkage blocks promoted by homozygosity (Tanck *et al*., 2001).

To exploit the residual variability present within the inbred population, parental lines were screened for high temperature tolerance using TIR. Approximately 2% of the surviving seedlings developed into plants in the field.

The selected parental lines established in the field showed higher membrane integrity when exposed to methyl viologen and high temperature under high light conditions (Fig. 5). The selected thermotolerant parental lines were crossed and the F_1 hybrid seeds obtained were assessed for thermotolerance both at the seedling and at the plant level. The selected hybrid showed increased recovery growth both at the seedling as well as at the plant level on exposure to stress (Figs 6, 8). The tolerance to oxidative stress induced by Menadione and methyl viologen was high in the selected hybrids. In addition, under oxidative stress, recovery growth and maintenance of membrane integrity was high. Results of the present study and earlier findings in peas (Srikanthbabu *et al*., 2002), sunflower (Kumar *et al*., 1999; Mamatha Reddy, 2000) and in groundnut (Gopalakrishna, 2001) confirms the potentiality of the TIR technique to identify stress-tolerant lines within either an open-pollinated variety or an inbred population.

Expression of HSPs

The functional relevance of HSPs has been convincingly demonstrated by functional genomics approach (Lee and Schoffl, 1996; Malik *et al*., 1999), through mutant analysis (Burke *et al*., 2000; Hong and Vierling, 2000). The significance of HSP 104 has been well documented by complementation studies with soybean and *Arabidopsis* HSP 104 in yeast mutants with an impaired capacity for HSP 104 (Sanchez and Lindquist, 1990; Parsell *et al*., 1994; Lee *et al*., 1994; Schirmer *et al*., 1994). Even the yeast HSP 90 mutants were highly thermosensitive (Borkovich *et al*., 1989). In these studies, the selected hybrid identified by the TIR technique showed enhanced expression of HSP 90 (Fig. 9B) and HSP 104 (Fig. 9C). This evidence indicates that several HSPs are up-regulated that could be because of the efficiency of upstream regulatory mechanisms like stress perception and transduction leading to the expression of transcription factors. Hence the expression of HSFs was studied in selected hybrids.

The regulatory proteins, HSFs, are structurally conserved throughout eukaryotic organisms (Scharf *et al*., 1998) and exist as inactive proteins mostly found in the cytoplasm. Stress causes activation with oligomerization and recompartmentation to the nucleus and binding to the promoter target sequences triggers transcription of the heat stress genes. There are at least three different HSFs, which belong to two subfamilies ($HSFA_1$, $HSFA_2$ and $HSFB_1$), two of them are heat-inducible proteins themselves (Scharf *et al*., 1998). An extended HR-A/B region in the subfamily A and the heat-dependent expression are unique features of the plant HSF system (Bharti *et al*., 2000; Scharf *et al*., 1998). In tomato, HSF A_1 is constitutively expressed whereas $HSFA_2$ and $HSFB_1$ are heat shock-inducible proteins (Scharf *et al*., 1998).

To examine the expression pattern of HSFs, antibodies were developed against a 21 amino acid sequence in the HR A/B oligomerization domain. Since HR A/B of the oligomerization domain is specific to HSFA, the antibodies will recognize the HSFA group of proteins. The western analysis indicated that HR A/B cross-reacted with only one protein with the molecular weight of 55 kDa. Since the antibodies developed against heptad repeats of A/B the HSF that is expressed in sunflower upon exposure to stress

belongs to HSFA and based on the molecular weight it is predicted to be $HSFA_2$.

The results showed that TIR is a potential technique to identify thermotolerant variants within cultivars or even in the inbred population. The F_1 hybrid developed from the thermotolerant variants of parental lines showed higher temperature tolerance and oxidative stress tolerance. The enhanced thermotolerance of the selected hybrid was associated with a higher expression of high molecular weight HSPs and the heat stress transcription factor HSFA.

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