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Original Research Article

Study of Genetic Diversity and Identification of Soluble Protein Markers for Cold Tolerance in Mungbean

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A B S T R A C T

A set of fifty nine mungbean germplasm accessions including popularly adapted local land races, important breeding lines and standard ruling varieties were screened for cold stress tolerance at seedling stage. Four genotypes e.g., OUM 132 Sel.,SML 668, OBGG 229 and OBGG 52 have been identified to possess cold tolerance. Total soluble protein profiling was carried out at seedling stage in 18 selected mungbean genotypes comprising four cold tolerant, four cold sensitive, five cold tolerant and five moderately cold tolerant mungbean genotypes to explore differentially expressed polypeptides in response to artificially induced cold stress. Seed protein profiles revealed nine scorable polypeptide bands with molecular weights ranging from 3.2 to 98.0kD. A specific 48kD polypeptide band was present in all cold sensitive test genotypes and it was absent in cold resistant genotypes (OUM 132 selection, SML 668, OBGG 229 and OBGG 52). Hence, it couid serve as an indirect marker for screening genotypes for cold tolerance. Besides, the genotype specific polypeptide pattern observed in the present pursuit may be used for varietal identification.

Keywords

Genetic diversity, Soluble protein profile, Cold tolerance, Mungbean (*Vigna*

Introduction

Mungbean (*Vigna radiata* (L.) wilczek, 2n= 22) is an important short duration pulse crop of India and Odisha in particular. Generally, pulses are very susceptible to cold stress (Tripathy *et al.*, 2011). Unfortunately, available mungbean varieties lack satisfactory level of cold tolerance and at present cold tolerant high yielding varieties are not available in this crop. The extent and rate of progress in improving cold stress tolerance through conventional breeding is limited owing to its multigenic nature and complex mechanism involved. Therefore, searching for candidate genes conferring cold tolerance could be a practical proposition. In this context, screening of a large collection of germplasm lines including wild accessions can pave the way for identification of tolerant genotypes. Cold tolerant genotypes are reported to accumulate stress related proteins to combat low temperature during growth stages. Russouw *et al.*, (1995) isolated and characterized an 11kD LEA(late embryogenic abundant) like-protein from embryonic axes during development of pea seeds. Besides, a few low molecular weight polypeptides associated with responses to

water deficit was observed at 22kD for *Lathyrus sativus* (Tyagi *et al.*, 1995). 26kD osmotin in tobacco (Singh *et al.*, 1987) and 23kD polypeptide in rice (Rao *et al.*, 1993). Polypeptide banding pattern has been also effectively employed by Afiah and Rashed (2000) and Sinha *et al.*, (1999) for isolation of plants under calcareous soil in mungbean and for tolerance to salt stress in *Lathyrus sativus* (L.) respectively. Abdellati *et al.*, (2012) differentiated drought tolerant varieties from susceptible genotypes of faba bean by polypeptide profiling. In the present investigation, the authors attempted to unravel polypeptides markers associated with cold tolerance using soluble protein profiling of mungbean.

Materials and Methods

A large collection of 59 mungbean genotypes were phenotyped for status of cold tolerance at seedling stage. Nineteen selected mungbean genotypes comprising cold tolerant and sensitive checks were field tested. Total seed storage protein of matured seeds of each of these genotypes was extracted with extraction buffer (50mM Tris-Cl pH 7.8, 5mM β- Mercaptoethanol and 5mM EDTA), denatured with an equal volume of cracking buffer (0.125M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.1% Bromophenol blue) at 80^{o}C in hot water bath. Seed proteins were analysed simultaneously by running two gels at a time in a vertical slab gel (12.5% polyacrylamide gel) at constant current of 60mA (2.5mA per lane for two gels run each time) for four hours following Laemmli (1970) with minor modifications. Reproducibility was confirmed by minimum of two repeats of each run of sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) under similar electrophoretic conditions. After electrophoresis, gels were stained with 0.125% w/v coomassie brilliant Blue R 250, 50%v/v methanol, and 10% v/v glacial acetic acid for four hours with intermittent shaking followed by destaining overnight in 50% methanol and 10% glacial acetic acid; and finally, several washings with 5% methanol and 7% glacial acetic acid. The molecular weights of the dissociated polypeptides were determined by using molecular weight marker with known molecular weights i.e., bovine plasma albumin, (66kD), egg albumin (45kD), glyceraldehydes-3-phosphate dehydrogenase (34.7kD) and bovine pancreas tripsinogen (24kD). Differentially expressed unique polypeptide bands were noted to find relationship with cold tolerance.

Results and Discussion

Low temperature, Drought and high salinity are some of the environmental limitations that have adverse effects on plant growth and crop productivity. Plants respond and adapt to these stresses to survive under stress conditions at cellular and molecular levels as well as physiological and biochemical levels. Expression of a variety of genes is induced by these stresses. The product of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress responses (Shinozaki *et al.*, 2003).

The genes may encode some specific enzymes or membrane proteins that alter membrane properties, and enzymes involved in production of osmoprotectants and products involved in signal transduction of stress responses. At the regulation of gene expression level, stress inducible promoters, such as, rd29A has been identified in tobacco which led to over-expression of stress inducible gene (DREB1A) under drought and low tempterature (Kasuga *et al.*, 2004).

Different species vary in their inherent mechanism to combat stress situations. Isolation of cDNA clones encoding stress inducible gene(s) following full length micro array and gene chip array could be a proper strategy for analyzing the molecular basis of stress tolerance. Molecular screening of genotypes for cold tolerance is considered rapid and reliable as compared to scoring based on phenotypic appearance under stress condition in the field. Identification of stress inducible polypeptides and isozymes elicited during developmental stages(seedling and vegetative stages) under cold stress could be safely used to elucidate genotype- specific stress response. The polypeptide banding pattern based on SDS-PAGE of total soluble protein does not require amplification as in case of random amplified polymorphic DNA (RAPD) or radio-isotope/ fluorescent labeling as in case of restriction fragment length polymorphism(RFLP). In spite of the fact that, the degree of intra-specific variation exhibited by polypeptide banding pattern in SDS-PAGE is lower as compared to RAPD and RFLP, this technique could be used to detect specific polypeptide marker(s) for cold tolerance.

SDS-PAGE of total soluble proteins generates different profiles comprised of several polypeptide sub-units which migrate in the gel according to their molecular weights. The number of such sub-units indicates the number of multigene families involved(de Lumen 1990). Mutation in these gene families or their regulator genes leads to deletion of some or all the genes or production of new alleles which forms the basis of variation in the polypeptide banding patterns involving presence or absence of bands. In addition, polymorphism in terms of intensity of bands could provide information in shutting of some members of the multigene family that could produce few copies in the genotype exhibiting faint bands. Electrophoregrams of a fairly large number of genotypes may also reveal distinct alleles for gene families comprising two molecular variants of a polypeptide sub-unit under monogenic control. Such polymorphic polypeptides with varying molecular weights are considered as polypeptide markers.

The polymorphism of polypeptide markers are in vogue used for characterization and categorization of genotypes in addition to its use in hybrid selection, marker assisted selection, elucidation of genetic control of protein expression, linkage of polypeptide bands, stability of polypeptide banding patterns, genome homology, center of genetic diversity and evolutionary pathways.

Out of the total fifty nine genotypes tested for cold tolerance in a growth chamber maintained at 8^0C with 72% relative humidity and 12h photoperiod each day(Fig 1a), five genotypes e.g, OUM 132 Sel.,SML 668, OBGG 229, OBGG 52 and LGG 460 scored resistant(score: o)(Fig 1b), five genotypes e.g., Dhauli, TARM 2, TM 94-12, T 7-10 Sel. and Sudhasarangi local-C scored tolerant (Score: 1-3), five genotypes e.g.,TARM 1, Mayurbhanj local(urdbean), Samarjhola local, OGG 57 and Raipur local appeared as moderately tolerant(Score: 4-5), 22 genotypes exhibited moderately susceptible (score: 6-7) and rest of the varieties were recorded as susceptible (score: 8-9) to cold stress.

Typical symptoms of cold stress injuries noticed were arrest of growth of apical meristem, wilting due to loss of turgidity of leaf tissue, drying of leaflets and whole seedling due to desiccation. Root and shoot fresh weights of cold acclimated seedlings decreased as compared to non-acclimated seedlings under cold stress. Root and shoot

growth (length) seemed to be arrested under prolonged cold stress. Dry weight of root was much reduced than shoot under cold stress than unstressed or control seedlings irrespective of the test genotypes.

Total soluble protein was extracted from leaf sample of seedlings after a month of erstwhile mentioned cold stress treatment. SDS-PAGE of total soluble protein of two sets of genotypes was carried out simultaneously by running two gels at a time in a vertical slab gel apparatus under similar electrophoretic conditions (220v constant voltage, 36mA constant current at 4^0C). The first set of genotypes comprised four cold resistant and four cold sensitive genotypes; and the second set included five cold tolerant and five moderately cold tolerant varieties of mungbean (Table 1). The SDS-PAGE revealed altogether nine scorable polypeptide bands (B_1 to B_9) with molecular weights ranging from 3.2 to 98kd (Table 1 and 2). This evidenced presence of at least nine multigene families involved in expression of total soluble protein under cold stress. For ease of detection of bands, four distinct zones of polypeptide migration could be assigned in the soluble protein profile i.e., A(≥ 90kd), B(≥40-90kd), C(≥ 15-40kd) and D(≥ 3.0-15kd).

The total soluble protein profiles under cold stress revealed considerably high polymorphism in terms of presence (+) or absence (-) of polypeptide bands as well as intensity of bands for different test genotypes. In the present investigation, three monomorphic bands were recognized with molecular weights 28.7, 16.0 and 3.2kd out of nine polypeptide bands scored. Thus, the soluble protein profile showed 66.66% polymorphism. The intensity of bands could reveal the quantitative variation in expression of polypeptides under cold stress. The presence of densely stained bands were marked bold + (Table 2) to discriminate genotypes for ease of varietal characterization.

Out of three monomorphic bands, band B7(28.7kd) and band B8(16kd) exhibited dense band intensity irrespective of the test varieties including mayurbhanj local(urdbean) analysed for SDS-PAGE. Thus, these bands could serve as characteristic features of *Vigna* species. Altogather 11 protein types have been recognized among the eighteen test genotypes based on presence (+) and absence (-) of polypeptide bands. Protein type-1 included Bhwanipatna local 2B, Mayurbhanj local and Samarjhola local. These genotypes revealed all the nine polypeptide bands, but yet they differ in terms of intensity of bands. Protein type -2 to 6 included two varieties each, such as, OUM 132 Sel.& SML 668; OBGG 52 & TARM 2; TM 94-12 & T 7-10 Sel.; Dhauli & Raipur local; and Sudhasarangi local-C & OGG 57 respectively. Protein type 7 to 11 contained single variety each, such as, OBGG 229, Dayapalli local, Hum 3 Sel., ML 613 and TARM 1 respectively. These varieties exhibited genotype-specific protein banding pattern. Such information would be helpful in varietal identification in seed production and could serve as a valuable criterion for DUS testing. Polypeptide banding pattern also allows detection of species –specific protein markers for *Cajanus cajan* and *Cajanus cajanifolius* (Panigrahi *et al.*, 2001).

Among different electrophoretic bands scored, B6 (48kd) is very specific to sensitive varieties only in the first set of genotypes analysed for SDS-PAGE. Such a polypeptide band was absent in cold resistant genotypes(OUM 132 selection, SML 668, OBGG 229 and OBGG 52) and hence it couid serve as an indirect marker

for screening genotypes for cold tolerance. Deletion mutation in regulatory and structural genes may lead to failure of protein expression.Besides, absence of a polypeptide band could be attributed to decrease in polysome content and specific changes in poly(A) RNA populations which are general responses in plants exposed to low water potential either through drought or cold. Such differentially expressed polypeptides present only in the susceptible genotypes were detected for indirect screening of YMV disease resistance in mungbean (Patnaik and Kole 2002) and green leafhopper resistance in rice (Padmavathi *et al* 1999). Sinha *et al* (1999), however, reported significantly reduced intensity of several low molecular weight polypeptide bands (50, 45, 40, and 14kd) in SDS-PAGE protein profile of NaCl stress induced seedlings of *Lathyrus sativus* L. which mean salt stress elicites some polypeptides. They also reported similar response for high molecular weight polypeptides (82, 74 and 42kd) only under prolonged water stress in *Lathyrus.* A 43kd dehydrin -like protein band was visible in cold acclimated leaf of olive cultivars with different band intensities and such a dehydrin-like protein is reported to exhibit seasonal fluctuations related to cold-hardiness in olive (Cansev *et al* 2008).

The response of dehydration tolerance also reported to involve down regulation of several genes in *C. plantagineum*. Some of the transcripts encoding proteins relevant to photosynthesis are down regulated during dehydration process and thus possibly reduce the photo-oxidative stress. Jiang *et al*.(1995) have also shown that the promoter regions of storage protein genes contain the information for their down regulation during seed desiccation.

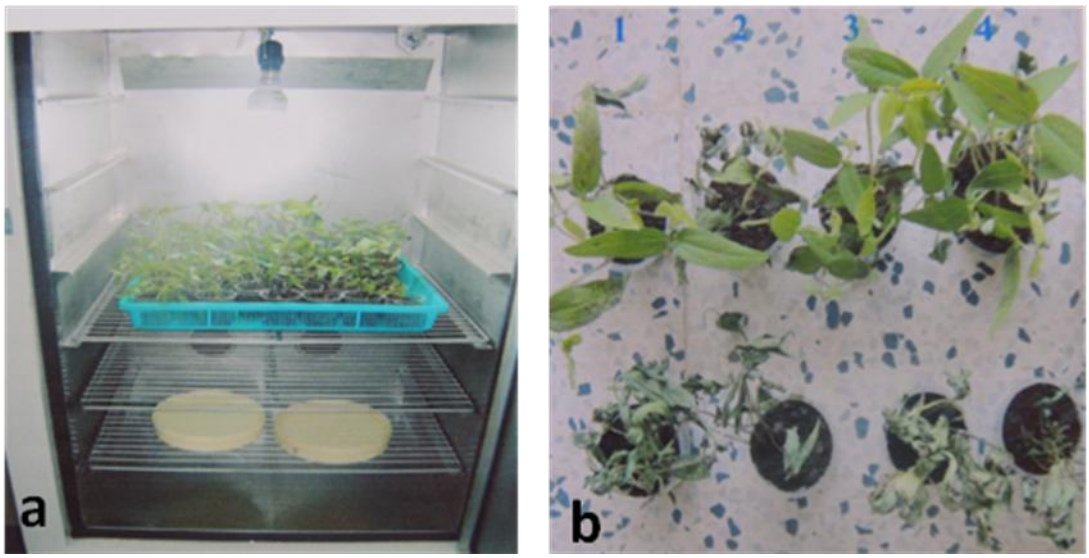
Table.1 List of test genotypes for SDS-PAGE of total soluble proteins

Sl. No.	Genotype	Status of cold tolerance	Seed colour
1.	OUM 132 selection	Resistant	Glossy black
2.	SML 668	-do-	Brownish green
3.	OBGG 229	-do-	Glossy green
4.	OBGG – 52	-do-	Green
5.	Dayapalli local	Sensitive	Glossy brownish green
6.	HUM-3 Selection	-do-	Green
7.	Bhawanipatna local – 2B	-do-	Dull black
8.	ML 613	-do-	Deep green
9.	Dhauli	Tolerant	Green
10.	TARM-2	-do-	Green
11.	TM 94 – 12	-do-	Green
12.	T 7-10 selection	-do-	Glossy green
13.	Sudhasarangi local – C	-do-	Dull black
14.	TARM-1	Tolerant	Green
15.	Mayurbhanj local	-do-	Dirty black
16.	Samarjhola local	-do-	Glossy green
17.	OGG 57	-do-	Green
18.	Raipur local	-do-	Green

Table.2 Electrophoretic (SDS-PAGE) polypeptide banding patterns of total soluble proteins of 18 test genotypes

Band No.	Mol. Wt.(kd)	OUM 132 Sel.	SML 668	OBGG 229	OBGG 52	Dayapall Local	HUM3 Sel.	Bhawanipatna Local-2B	ML 613	Dhauli	TARM 2	TM 94-12	T 7-10 Selection	Sudha-sarangi Local-C	TARM 1	MayurBhanj Local	SamarJhola Local	OGG 57	Raipur Local
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
B1	98.0	+	+	-	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-
B2	96.6	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+	+	+	-
B3	92.3	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
B4	77.5	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+
B5	60.0	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
B6	48.0	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-
B7	28.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B8	16.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B9	3.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig.1a Cold stress treatment (8^{o}C, RH 72%, 12h photoperiod) to 59 test genotypes of munbean; **b** Cold tolerant genotypes (top row 1 to 4): OUM 132 Sel., SML 668, OBGG 229 and OBGG 52; Cold sensitive genotypes (bottom row 1-4) : Dayapalli local, HUM 3 Sel., Bhawanipatna local 3B, ML 613



Recently, it is understood that H_1 proteins accumulates in response to dehydration in vegetative tissues of tomato and in turn these repress the expression of drought responsive genes. Hashimoto and Komatsu (2007) carried out 2-D electrophoresis of total crude proteins from two week old rice seedlings and indicated that out of 250-400 protein spots, 39 proteins changed in abundance after cold stress, with 19 proteins increasing and 20 proteins decreasing. The proteins related to energy metabolism and stress related proteins were up-regulated while defence related proteins were down regulated and even disappear under long-term cold stress.

The physiological implication of the poly peptide expression only in the susceptible genotypes in the present study and the strength of linkage of the loci controlling the polypeptide (48kd) and sensitivity to cold stress are not known. However, the present molecular identification of cold stress related responses of mungbean cultivars may provide markers for selection in breeding programme and genes for improvement through transgenic technology.

The results generated from SDS-PAGE soluble protein fractions under cold stress were used for drawing the genetic relationships among the 18 mungbean genotypes. The similarity indices were estimated for each pair-wise group using UPGMA computer software programme and are presented in Table 2. The highest similarity value (1.00) was observed between OUM 132 Sel and SML 668; OBGG 52 and TARM 2; TM 94-12 and T 7-10 Sel.; Bhawanipatna local 2B and Mayurbhanj local; Bhawanipatna local 2B and Samarjhola local; Dhauli and Raipur local; and Sudhasarangi local C and OGG 57 indicating that these paired lines might have close homology at genomic level and thereby exhibit close relationship in gene expression in terms of polypeptide banding pattern. This is in conformity with the erstwhile mentioned protein types recognized in the present investigation as each of these paired genotypes has shown to have specific protein type.

The lowest similarity value (0.38) was recorded between OBGG 229 with either of the genotypes e.g., OUM 132 Sel., SML668 and ML 613 indicating that OBGG 229 is quite genetically distant from either of the above three genotypes. Crosses particularly OBGG 229x OUM 132 Sel and OBGG 229 x SML 668 that involve cold tolerant varieties may excel in achieving further higher level of cold tolerance. Besides, a cross between OBGG 229(resistant) x ML 613(susceptible) can also result better recombinants for genetic improvement of cold tolerance.

Cluster analysis is a valuable tool for subdividing genotypes into groups including similar and dissimilar lines. A dendrogram was constructed to show genetic relationships among the test genotypes. Eighteen genotypes were grouped into four broad groups/clusters at S.I value 0.68. Cluster-1 contained two genotypes (OBGG 52 and TARM 2). OBGG 229 constituted a single variety cluster and was placed in Cluster-2. Cluster -3 and 4 were multi-variety clusters. Cluster-3 contained four genotypes(ML 613, HUM 3 Sel., Dayapalli local and TARM 1) and Cluster- 4 included rest 11 genotypes.

At highest phenon level(100%), the above multi-variety clusters were further dissociated into sub-clusters each containing either a single or few genotypes. Altogether 11 genetic groups were formed each with specific polypeptide banding pattern. Thus, the clustering pattern confirms the erstwhile mentioned categorization of genotypes into protein types based on presence(+) and absence of polypeptide bands.

The presence of a dense band (B4: 77.5kd) in OBGG 229 is clearly distinguishable from rest of the genotypes. Similarly, the absence of band B5 (60kd) in OBGG 229 and Dayapalli local in their soluble protein profile made them distinct from rest of the varieties. SDS-PAGE is, thus, a simple but useful technique which can also be used for identification and characterization of mungbean varieties.

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