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Research Information

Cellular and molecular characterization of resistance to *B. sorokiniana* in bread wheat

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Abstract

Spot blotch disease of wheat has now been considered as one of the major constraints in wheat growing regions specially in South East Asia and Latin American countries where warm humid conditions persist during wheat crop season (Kumar et al. 2002). Due to tremendous breeding efforts, new partially resistant lines have recently been released. However, lack of complete resistant genotypes and insufficiency of knowledge about the resistance mechanism(s) still hamper sustained breeding efforts. We comparatively investigated the interaction of *B. sorokiniana* with moderate resistant genotype Yangmai 6 and susceptible genotype Sonalika. Cytological data have been recorded at different time points about the changes occurring at cellular level viz., cell wall-associated defense (CWA), hypersensitive reaction (HR), and post-penetration hypersensitive reaction (PPHR). The molecular studies aim at identification and functional analysis of genes, whose expression is getting influenced by the onset of *B. sorokiniana* infection. On the basis of *macroarray* spotted with 1,536 cDNA fragments, hybridized with probes prepared from barley leaves at two time points after fungal infection, 52 candidate genes were identified either up- or down regulated. Five randomly selected candidate genes of this collection were further verified by RT-PCR and altered candidate gene expression was compared at mRNA transcriptional level in response to *B. sorokiniana* infection in both genotypes. The candidate genes were actin, ABC transporter related gene, ferritin, Mg-chelatase subunit XANTHA-F and RNA binding protein Rp 120. The putative role of the candidate genes will be discussed.

Key words: Wheat, *Bipolaris sorokiniana, macroarray*, RT-PCR, candidate genes

Introduction

Wheat is considered as most widely grown and consumed food crop of the world and is expected to be the staple food of around 35% of the world population (Pingali ed. 1999). The present wheat production is about 560 million tonnes and to feed world ever-growing population in 2020 we have to produce from 860 to 1040 million tonnes with 1.6 to 2.6% annual growth rate. To achieve the target, it is essential to keep the wheat crop free from various biotic stresses including spot blotch (*B. sorokiniana*), which are limiting wheat production in different parts of the world (Mathur and Coufer 1993). Spot blotch has now been emerged as one of the major production constraint in the wheat growing areas particularly in South East Asia and Latin American countries where nearly 12 million hectares of land under cultivation are affected (Nagarajan and Kumar 1998). The pathogen affects almost all the crops belonging to Graminace family, though the chances of migration of isolate of one crop to other crop are remote (Bakonyi et al. 1997; Pandey et al. 2005). *B. sorokiniana* causes foliar spot blotch, root rot, black point on grains, head blight and seedling blight of wheat and barley (Kumar et al. 2002). Estimates of yield losses due to spot blotch are reported to vary from 15.5 to 19.6% (Dubin and Van Ginkel 1991), 20 to 80% (Duveiller and Gilchrist 1994), and may be upto 100% under severe infection conditions (Mehta 1994). An integrated approach including right cropping system, use of fungicides and introduction of new resistant varieties can provide effective control of spot blotch under field

conditions (Joshi and Chand 2002). *Bipolaris sorokiniana* is a hemibiotrophic fungus having both biotrophic and necrotrophic phases. The biotrophic growth phase is confined to a single epidermal host cell that is invaded by a network of infected hyphae, while the necrotrophic growth phase is characterised by invasion of the mesophyll tissue and host cell death (Kumar et al. 2002; Schäfer et al. 2004). Plant resistance against any pathogen can be classified broadly into two classes non-host resistance and host resistance. The non-host resistance is found in all cultivars of a plant species to all races of a certain pathogen. Non-host resistance obviously relies on a complex genetic control and implies a genotype of divergent defence components whose existence or induction does not depend on known resistance genes (Heath 2000). The molecular basis of non-host resistance includes synthesis of secondary metabolites with antimicrobial activity, hypersensitive reaction (HR), production of phytoalexins, extra cellular accumulation of reactive oxygen intermediates (ROI) and the activation of defence related genes especially, cell wall-associated defence (CWA) mechanisms, and post-penetration hypersensitive reaction (PPHR). Host resistance specific to genotype of the host, starts to work when pathogen over come non-host resistance devices. The increasing threat of spot blotch calls for serious efforts to understand various dimensions including cytological and molecular basis of the resistance mechanism to breed resistant genotypes. However, to reveal the resistance mechanism at cytological basis, still a detailed cytological study is needed to know about different components, which are directly or indirectly related to resistance mechanism. The aim of molecular studies about candidate genes along with cytological studies was just to bring valuable information to have the better understanding of resistance mechanism at cytological as well as molecular level.

Materials and methods

Genotypes and pathogen

Moderately resistant genotype Yangmai 6 and susceptible genotype Sonalika were used in the study. Seeds of the given varieties were obtained from International Maize and Wheat Improvement Centre (CIMMYT), Mexico and the Directorate of Wheat Research (DWR), Karnal, India, respectively. Informations about isolation and origin of Bs culture used in the study are given in Kumar et al. (2001). For the spray, bits from monoconidial cultures were placed on the filter paper in SNA media and incubated at room temperature under a 12 h-photoperiod. After 7 days, petri plates were flooded with the solution of distilled water with Tween 20 in the ratio (5000:1). Conidia were scraped out from the surface with a spatula and filtered through two layered muslin cloth before inoculation.

Microscopic analysis

For cytology, About 6 cm long leaf segments of flag leaves from 15-day-old plants were placed on whatman filter paper and incubated with spore suspension containing 50,000 spores/ml. After spraying with spore suspension, leaf segments were placed on 0.5% water agar (40 mg/l bengimidazol) in a plastic dish (>95% RH) closed tightly with a lid. Plates were incubated in a growing chamber (24˚C day/night and 16-h photoperiod). The leaf segments were evaluated according to the stipulated time point kinetics viz., 20, 40 and 60 hours respectively. Calcofluor has been used for cell and hyphal wall detection (Rohringer 1977; Schäfer et al. 2004) and facilitates the location of fungal penetration sites in leaf tissue. For better contrast of defense reactions, histochemical detection of H_2O_2 was carried out by an endogenous peroxidase-dependent *in situ* staining procedure with 3,3-diaminobenzidine (DAB, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Thordal-Christensen et al. 1997). Leaves were infiltrated with 1 mg/ml DAB dissolved in water (pH3.8, HCl) and injected into wheat first leaves 2 h before fixation. Leaf fixation and microscopy were conducted as described by Hückelhoven and Kogel (1998). Whole cell and sub cellular autoflorescece was observed under epifluorescence microscopy (Kumar et al. 2002; Schäfer et al. 2004). Moreover, calcofluor stained hyphae were observed by florescence microscopy. Four leaf segments and 60-70 interaction sites on each leaf segment were elucidated in two independent experiments.

Identification and functional analysis of candidate genes

For molecular analysis, plants were grown in a growth chamber at 24˚C/18˚C (day/night cycle), 60% RH and a photoperiod of 16h (240 µmol $m^{-2}S^{-1}$ photon flux density). 15 days old plants were inoculated by spraying a spore suspension containing 100,000 spores/ml. An epidermis-specific cDNA library derived from barley leaves treated with culture filtrate of *B. sorokiniana* was synthesized. On the basis of macroarray spotted with 1,536 cDNA fragments, hybridized with probes prepared from barley leaves at two time points after fungal infection, 52 candidate genes were identified either up or down regulated. Five randomly selected candidate genes of this collection were further verified by RT-PCR and altered candidate gene expression was compared at mRNA transcriptional level in response to *B. sorokiniana* infection in wheat.

RNA extraction and cDNA synthesis

Table 1. Name of the clone, primer sequences, annealing temperature, amplification cycles and expected product size

Clone	Sequences of primers	Annealing temp (^{o}C)	Amp cycles	Product size (bp)
GBN002P06	5`-AGAAGGCGAAGAAGGAAAGG-3' 3`-TTGAGCTTAGGGGAAAAACAA-5`	52	39	269
GBN003B04	5′-CAGGTTGCACGTAGATGAGC-3′ 3′-GAGTGCCCCACCAAAGAATA-5′	58	27	291
GBN003B06	5′-TAAGAGGGTGGCACATCTCC-3′ 3′-GGGCAAGGGAAGATGTAGGT-5′	58	27	154
GBN003K10	5′-ATCCGGCAATACAGTCAACA-3′ 3′-ACCGCCTGAGAGGAAGTACA-5′	54	27	250
GBN004053_C05	5′-GCCATGAAGAGTGCCAAGAT-3′ 3′-GCTCTTCCAGTTCAGCAACC-5′	56	34	340

Total RNA was extracted from 8 cm long leaf segment of flag leaf using RNA extraction buffer (Peq lab, Erlangen, Germany). RNA was extracted from the Bs treated leaf samples collected at the different time points viz., 0, 8, 24, 48 and 72 h after inoculation in parallel with corresponding mock treated leaf segments (1:5000; Tween 20:water). RNA quantity and quality was measured at 260 and 280 nm by UV-spectrophotometer (DU7400, Beckman) and in dehydrated formaldehyde gel electrophoresis, respectively. RT-PCR kit (MBI-Fermentas, Germany) was used for the synthesis of *cDNA* following the manufacture's instructions as written in the literature provided with Reverse Transcriptase enzyme. 4 µg RNA was used to construct the first strand cDNA by using Reverse transcriptase and oligo-DT primers. In the RT-PCR analysis, barley ESTs corresponding to these genes were used in the construction of primers for RT-PCR analysis (Table 1).

Results

Initial field screening

Both the varieties had been intensively screened under the field condition by the first author at the Agricultural Research Farm, Banaras Hindu University, Varanasi (India) during 1999 to 2001. Yangmai 6 was showing less disease severity even at early dough stage (growth stage 83; Zadoks et al. 1974) (data not shown). In addition to this, both varieties were also screened under controlled conditions by producing artificial epiphytotic condition at Institute of Phytopathology and Applied Zoology, JLU, Giessen. Detached leaf segments of the youngest and 2nd youngest leaf from 15-day-old seedlings were screened under artificial epiphytotic condition to access the level of disease severity. Sonalika was showing more number of spot blotch lesion per leaf segment in comparison to moderate resistant genotype Yangmai 6.

Microscopic analysis

Fungal penetration through anticlinal cell walls and invasion of the mesophyll tissue starting from the epidermal layer were similar to what was found in barley-*B. sorokiniana* interaction in earlier studies (Kumar et al. 2001; Schäfer et al. 2004).

Fungal penetration into epidermal cells was termed "successful penetration" and invasion of mesophyll tissue "successful infection". Higher level of infection was observed in susceptible cultivar Sonalika. All defense responses were grouped into two broad classes i.e., Pre defense and post defense responses. Cell wall appositions (CWA) and hypersensitive reaction (HR) were considered under pre defense responses, while post penetration defense reaction (PPHR) was taken as post defense response. Higher frequency of non-penetrated epidermal CWA was observed in Yangmai 6 at all time points (20, 40 and 60 hai), while Sonalika showed higher frequency of penetration (Fig. 1).

The frequencies of non-penetrated epidermal cells that underwent an HR were analyzed. However, a rapid decrease at 40 and 60 h after inoculation was observed in moderate resistant genotype Yangmai 6. In the early hours (20 hai), the number of Post Penetration Hypersensitive Reaction (PPHR) sites was not too different in both varieties but at 40 hai, Yangmai 6 was showing comparatively higher level of PPHR in comparison to Sonalika (Fig. 1). The fungal spreading was observed to be correlated with H_2O_2

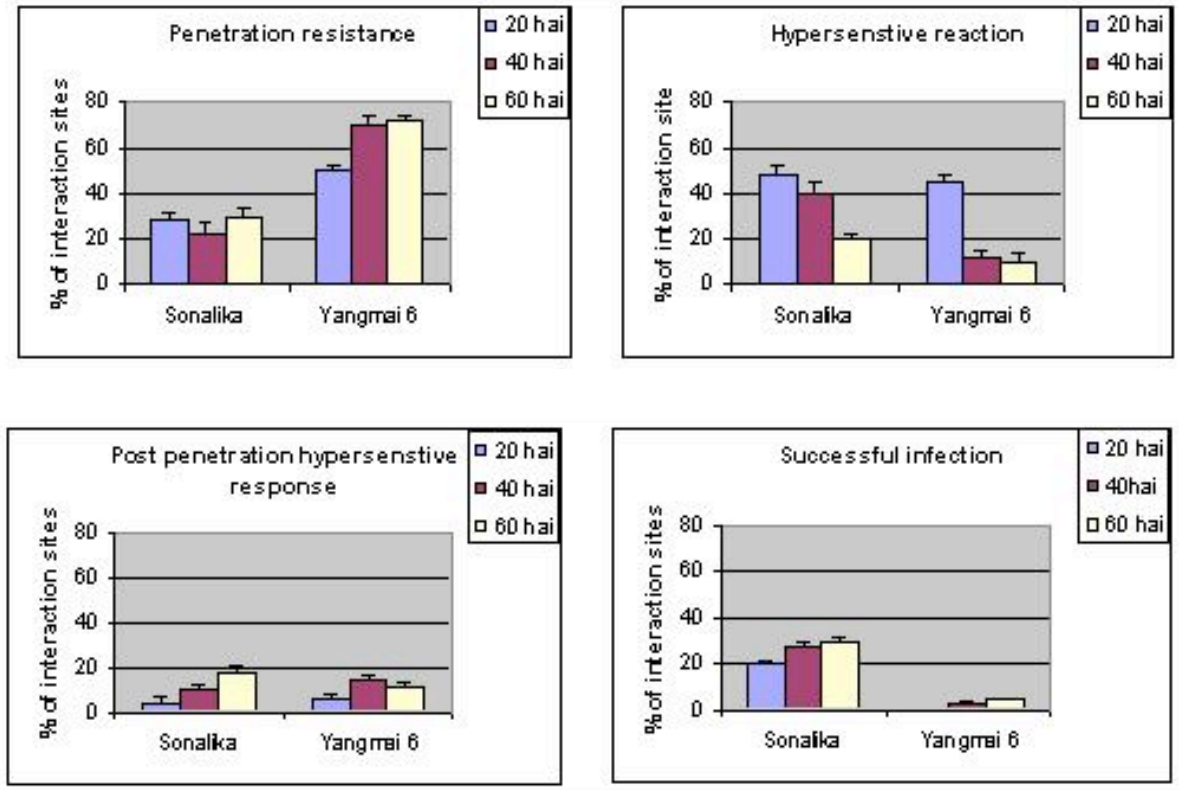

Fig. 1. Frequencies of penetration resistance, hypersensitive reaction, post penetration hypersensitive response and successful infection at 20, 40 and 60 h after infection in susceptible genotype Sonalika and moderate resistant genotype Yangmai 6 with Bs culture filtrate.

accumulation. The amount of H_2O_2 in the leaves showed a correlation with necrosis. Higher H_2O_2 accumulation was recorded in susceptible genotype Sonalika than Yangmai 6. Autofluorescence observations were closely related with subcellular and whole cell accumulation of H_2O_2 as visualized after the injection of 3,3' diaminobenzidine (DAB) into the excised leaf segments (Kumar et al. 2001).

Macroarray analysis with *Bipolaris sorokiniana*

To study the mechanism of resistance against spot blotch invasion, molecular study was began with macro array analysis based on barley crop. 52 genes were identified either up or down regulated at 20 and 40 h after inoculation with Bs culture. Reliable data were obtained by repeating array hybridization three times. Each clone was spotted two times on each array and mean values of the corresponding spots were used in signal transduction. The putative function of each gene was defined according to Blast X2 results (Schäfer 2004). Five of 52 randomly selected candidate genes were further verified by RT-PCR and altered candidate gene expression were compared at mRNA transcriptional level in response to *B. sorokiniana* infection in moderate resistant genotype Yangmai 6 and susceptible genotype Sonalika of wheat (Table 2).

RT-PCR analysis of candidate genes

In comparison to control, gene from *ABC1* family was showing down regulation at 8, 24 and 48 hai in Bs treated samples in both genotypes, Sonalika and Yangmai 6. However, at 72 hai bands of control and treated one were showing equal intensity in agarose gel having ethidium bromide (Fig. 2). The RT-PCR results were in accordance to Macro-array analysis based on barley.

Mg chelatase subunit XANTHA-F gene, observed to be down regulated in Macro-Array analysis was showing clear down regulation at 8 hai in both cultivars of wheat. However, an up regulation after 3 days (72 hai) was observed in Sonalika.

In case of *actin*, it was down regulated at all defined time points *viz.,* 8, 24, 48 and 72 h after inoculation. However, it is seen that down regulation was more readable in susceptible cultivar Sonalika. *Actin* gene was considered to be down regulated in Macro Array analysis with the infection of *B. sorokiniana*.

Ferritin gene was observed up regulated in both varieties at 72 h after inoculation in comparison to control. Moreover, it was more up regulated in

Table 2. Details about all the five candidate genes after array evaluation based on their temporal expression pattern and putative function

Clone ID	Homology to TIGR-entry	Score and E-value to TIGR entry	Gene(Blast N)	Putative function	Score and E-value to Blast X2	Expression
GBN002P06	TC 66635	1635 (6.6 E-70)	RNA binding protein Rp 120	Protein translation	1478 (0.00 E+0.00)	Up regulated
GBN003B06	TC 66495	1005 (3.2 E-41)	ferritin	Iron storage protein	417 (E-115)	Up regulated
GBN003B04	TC82956	1938 (6.7 E-83)	Transporter Family-1	Membrane, transporter	143 (2.00 E-33)	Down regulated
GBN003K10	TC 76826	1320 (2.0 E-55)	actin	Cytoskeleton	750 (0.0)	Down regulated
GBN004C05	TC 77299	1960 (1.4 E-83)	Mg-chelatase subunit XANTHA-F	photosynthesis	2726 (0.0)	Down regulated

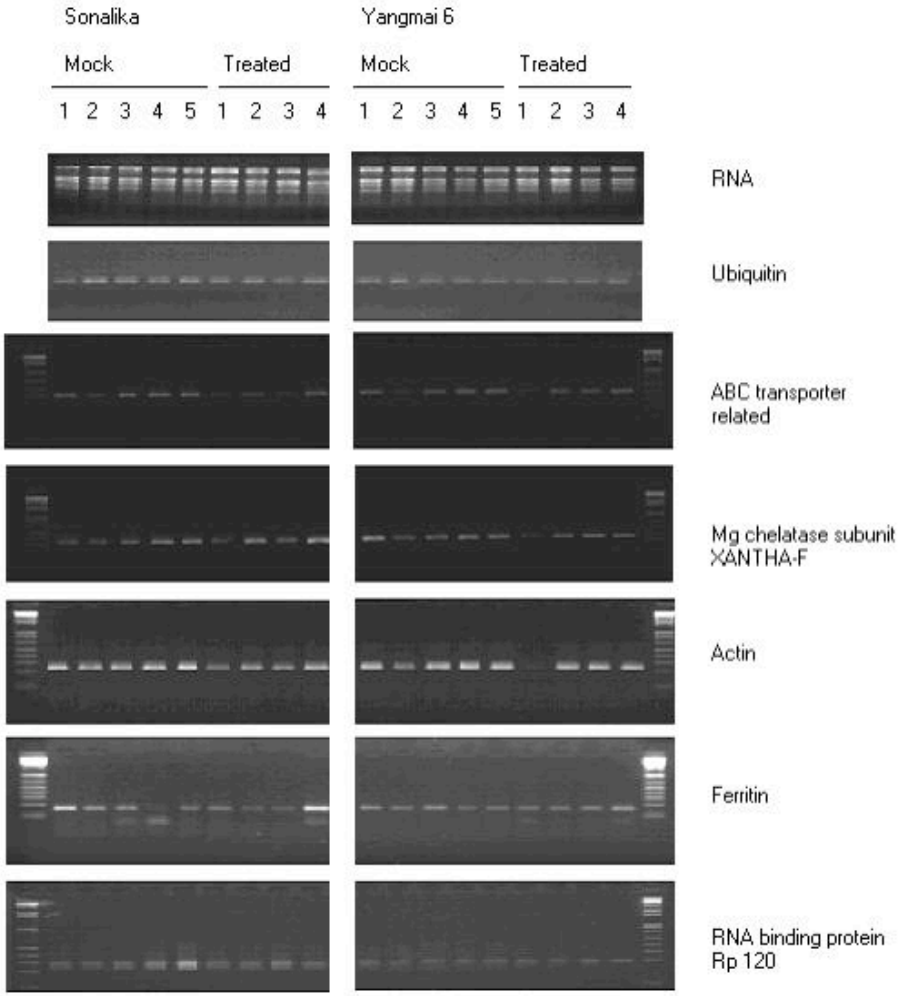

Fig. 2. RT-PCR analysis of differentially expressed gene transcripts after inoculation of wheat genotypes, Sonalika and Yangmai 6 with Bs culture. Transcript accumulation was analysed using total RNA extracted from leaves harvested at 0, 8, 24, 48 and 72 h after inoculation with Bs and mock-treated plants. RT-PCR products were detected in agrose gels stained with EtBr. Ubiquitin gene of barley was used to equalize the amount of cDNA for further analysis.

susceptible cultivar Sonalika than moderate resistant genotype Yangmai 6 with Bs culture filtrate. In both varieties, a down regulation at 24 h after inoculation was also noticed.

In contrast to Macro array analysis based on barley, *RNA binding protein Rp 120* was showing down regulation at 48 and 72 h after inoculation in wheat cultivar Sonalika compared to control (mock treated plants). However, slight up regulation was noticed at 48 and 72 h after inoculation in Yangmai 6.

Discussion

Cytological analysis

The purpose of characterization of disease resistance at cytological level was to build up a plate farm for molecular studies. Wheat resistance to *B. sorokiniana* is partially attributed to penetration resistance due to papilla-like CWA or early HR of non-penetrated cells (Fig. 1). Since, *B. sorokiniana* is a hemibiotroph (Kumar et al. 2002; Schafer et al. 2004), it takes food first from attacked living cell before the deposition of CWA and then from dead cell. Likewise, HR-associated cell death might be an inadequate measure to stop necrotrophic fungus (Kumar et al. 2001; Kumar et al. 2002). One of the acceptable reasons in the susceptible genotype might be that the activation of such defence responses often occurs so late that host fail to restrict ingress of the pathogen. Effective penetration resistance is closely associated with the local generation of H_2O_2 (Kumar et al. 2001; Shäfer et al. 2004). This H_2O_2 leads the necrosis of cells in the surroundings of pathogen and thus, localize the pathogen attack. Since, fungal penetration attempts into the epidermal cells were prevented as such at the site of H_2O_2 accumulation, therefore role of H_2O_2 in the inaccessibility of epidermal cells cannot be overruled. Moreover, post-penetration HR encapsulated the infected hyphae from first penetrated cell and therefore, localised the pathogen. Cell death at the early (epidermal) growth stage results in fungal arrest, while cell death at a later stage (mesophyll) might be either a prerequisite or a symptom of successful fungal development. More cell death symptoms in the mesophyll cell were observed in the susceptible genotype Sonalika. Relative decrease of HR particularly in Yangmai 6 can be explained by the fact that late penetration attempts failed more common in comparison to early penetration attempts like in Sonalika. While fungus continuously tried to penetrate from newly emerging appressoria during the entire time course of infection.

Candidate genes

The exact function of ABC1 family gene is not known. However, yeast ABC1 suppresses a cytochrome b translation defect and is essential for the electron transfer in the bc 1 complex. Sequence of our barley EST clone was showing similarity to the C-terminal end of Arabidopsis ABC1 line but was located away from ABC domain (250-300 bp) of Arabidopsis. It is suggested that members of ABC1 family are novel chaperones and unrelated to the ABC transporter proteins. The ABC1 family gene was observed down regulated at 8, 24 and 48 h after infection in Bs treated plants. Results were in close proximity with barley results based on MLO/mlo treated with *B. sorokiniana*.

Role of RNA binding protein Rp 120 is supposed to be associated with cytoskeleton as indicated by its sedimentation behaviour in sucrose density gradient and play a very active role during seed development (Sami et al. 2001). Most of ESTs in TIGR wheat library are found in developing seed or root libraries. However, presences of leaf ESTs in libraries (wlm 4) indicate a role of *RNA binding protein Rp 120* in the later growth phase of plant. Down regulation of this cytoskeleton-associated clone (GBN002P06) at 48 and 72 h after inoculation in susceptible genotype, Sonalika indicate that *B. sorokiniana* may alter or disrupt the cytoskeleton. However, slight up-regulation at 48 and 72 hai by resistant genotype Yangmai 6 may be taken as regaining of *RNA binding protein Rp 120* function.

GBN004C05 encoding for *Mg-chelatase subunit XANTHA-F* observed to be down regulated at 8 h after inoculation in both the varieties indicates that function of the gene is not linked with resistance reaction. Mg-chelatase, a multicomponent enzyme has a very crucial role in the chlorophyll synthesis pathway, owing to its position at the branch point between *haem* and chlorophyll synthesis. The actual role of *Mg-chelatase* catalases is the insertion of Mg into protoporphyrin IX. Down regulation may be taken as disrupt supply of Mg or ATPs requirements, since ATPs are required for activation as well as Mg^+ chelatation (Lee et al. 1992).

GBN003K10 representing the *Actin* gene of barley, showed a down regulation at all the time points viz., 8, 24, 48 and 72 h after inoculation compared to control in both the varieties of wheat. Moreover, down regulation was more readable in susceptible genotype Sonalika in comparison to Yangmai 6 (moderate resistant). *Actin* has a role in the development of cytoskeleton. It can be seen in the light that *B. sorokiniana* disrupt the basic structure of the cell.

Ferritin gene protects the plant against oxidative damage. Higher Ferritin transcriptional amount absorb more iron particles and protect the plants from the possible damage due to oxidative burst (Maria et al. 1999). *B. sorokiniana* like other pathogens also cause oxidative damage in the plants (Kumar et al. 2001). GBN003B06, clone for *ferritin* gene, showed a down regulation at 24 h and a up regulation at 72 h after inoculation with Bs culture in both varieties indicate

that pathogen causes initial oxidative damage in infected plant cells but then plant retrieve in the later growth phase of infection and try to overcome these oxidative damages. More up regulation of *Ferritin* at 72 hai in susceptible genotype might be seen in the light of more damages in susceptible genotype.

Study of *B. sorokiniana* at cytological level give us an initial idea to develop better understanding about different cellular barriers, play an important role in resistance mechanism. Moreover, knowledge about expression analysis of different candidate genes at Macro array and/or RT-PCR level can be used to improve the present status of disease resistance against *B. sorokiniana* by developing transgenic plants.

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Research Information

Relationship between carbon isotope discrimination, grain yield and water use efficiency in bread wheat under well-watered conditions

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Abstract

The relationship between carbon isotope discrimination (Δ), grain yield, and water use efficiency for biomass (WUE_B) and grain yield (WUE_G) were analyzed in a set of bread wheat (*Triticum aestivum* L. subsp. *aestivum*) genotypes including seven landraces as well as the Iranian cultivar Zakros. No significant relationship was found between Δ and grain yield. A significant negative correlation was noted between Δ, WUE_G and WUE_B. The Iranian landraces and the modern cultivar Zakros mainly differed for grain yield, harvest index and WUE_G. These results indicate that under well-watered conditions, Δ does not represent an accurate secondary trait for yield but can be used to estimate water use efficiency. They also suggest that WUE_G under well-watered conditions has been improved by modern selection through an increase of harvest index rather than of transpiration efficiency.

Introduction

The use of carbon isotope discrimination (Δ) has been proposed by several authors to select C_3 crops for grain yield and water use efficiency (WUE). In bread wheat (*Triticum aestivum* L. subsp. *aestivum*), Ehdaie and Waines (1993) reported a negative correlation between Δ and WUE while the relationship between Δ and grain yield was found highly dependent on the analyzed organ or tissue, the stage of sampling, and the environment and water regime (Monneveux et al. 2005).

The relationship between Δ, grain yield and WUE has been investigated in a set of Iranian bread wheat including seven landraces as well as the modern Iranian cultivar Zakros. Landraces still represent an important part of the 6.3 million hectares wheat area in Iran (Moghaddam et al. 1997). Little is known, however, about their efficiency to use available water.

Material and Methods

Plants were grown in a greenhouse, in plastic pots (one seedling per pot) filled with 1 kg of soil composed of 42% sand, 36% silt and 22% clay and placed in a randomized complete block design with three replications. Each pot was brought to water holding capacity. Pots were weighted every 2 or 3 day and amounts of water equal to the loss in weight were added until flag leaf yellowing. The total amount of water used was calculated as the difference between final and initial weight of the pot plus the total amount of water supplied to each pot and included both transpired and evaporated water (Ehdaie et al. 2003). Carbon isotope composition ($\delta^{13}C$) of grain samples was determined using an elemental analyzer isotope ratio mass spectrometer (University of Winnipeg Isotope Laboratory, Canada). $\delta^{13}C$ was calculated by comparing ^{13}C to ^{12}C composition of the samples (R_{sample}) relative to the composition of the Pee Dee Belemnite standard

Table 1. Carbon isotope discrimination, grain yield, total biomass, harvest index, and water use efficiency for grain yield (WUE_G) and total biomass (WUE_B) in landraces and the modern cultivar Zakros

	Δ (‰)	Grain yield (g per plant)	Total biomass (g per plant)	Harvest index	WUE_G (g kg^{-1})	WUE_B (g kg^{-1})
Iranian landraces	19.64a	2.63b	6.46a	0.41b	0.94b	2.31a
Zakros c.v.	19.23a	3.17a	7.05a	0.45a	1.06a	2.36a

Note. within columns, means followed with the same letter are not significantly at the 0.05 level

(R_{PDB}): $\delta^{13}C$ (‰)= [(R_{sample} / R_{PDB} −1) ×1000. Δ value of the samples was obtained according to the formula: Δ (‰) = (δ_a−δ_p)/(1+δ_p), where δ_p is the $\delta^{13}C$ of the plant sample and δ_a, the $\delta^{13}C$ of the atmospheric CO_2. δ_a was assumed to be −8 ‰ (Farquhar et al. 1989). At physiological maturity, aerial parts and roots were collected and dried separately. Grain yield per plant and total (aerial and root) biomass, were determined. Water use efficiency for biomass production (WUE_B) and for grain production (WUE_G) were calculated according to Ehdaie and Waines (1993) as total biomass per plant kg^{-1} water used and grain yield per plant kg^{-1} water used, respectively.

Results and Discussion

A wide variation was observed among accessions for Δ, which was in the range 18.60-21.20 ‰. No significant relationship was found between Δ, grain yield and total biomass (Fig. 1). A significant negative correlation was noted between Δ, WUE_G and WUE_B (Fig. 2). The Iranian landraces and the modern cultivar Zakros mainly differed for grain yield, harvest index and WUE_G (Table 1).

The lack of correlation between Δ and grain yield in bread wheat grown well-watered conditions has been already reported by Monneveux et al. (2004, 2005), Misra et al. (2006) and Xu et al. (2007). Under well-watered conditions, stomatal conductance is likely to be high in all cultivars resulting in increased C_i/C_a, the ratio of intercellular to atmospheric CO_2, and Δ values (Morgan et al. 1993), while increased photosynthetic capacity potentially decreases C_i/C_a. The decrease in C_i/C_a associated with increased photosynthetic capacity is consequently offset by the C_i/C_a increase resulting from stomatal aperture, hence reducing the possibility of association between Δ and grain yield.

The significant negative correlation noted between Δ, WUE_G and WUE_B is in good agreement with Knight et al. (1994) who reported negative associations between Δ and WUE in six field-grown crops, including bread and durum wheat, and with Ehdaie and Waines (1993) who found a negative correlation between Δ and WUE among bread cultivars from China, Iran and CIMMYT. According

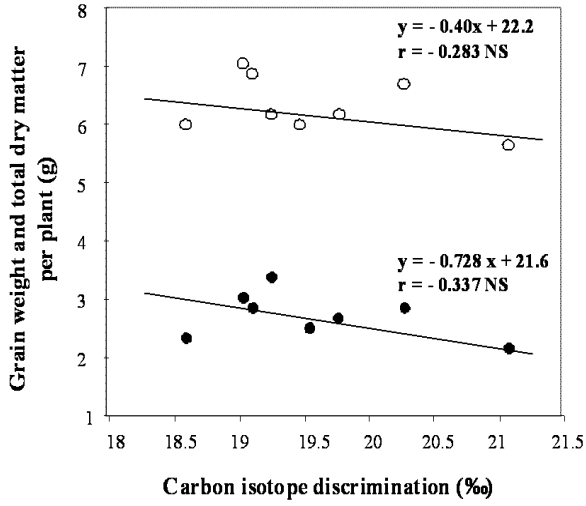

Fig. 1. Relationship between carbon isotope discrimination and grain weight and total dry matter per plant (NS; non significant)

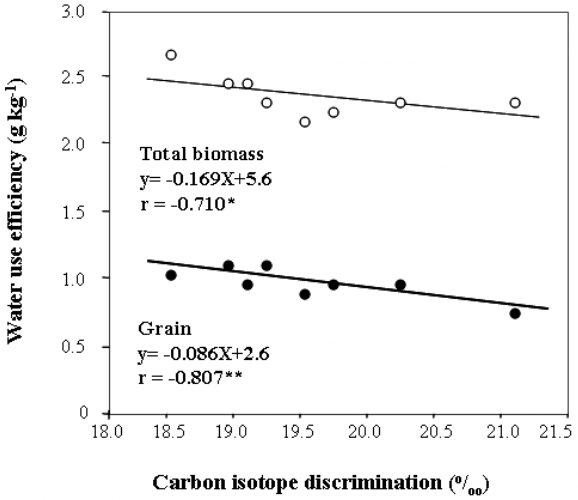

Fig. 2. Relationship between carbon isotope discrimination and water use efficiency for grain yield and biomass (*P<0.05; **P<0.01)

to Passioura (1977), higher WUE_G can be obtained by moving more of the available water through the crop (higher ratio of transpired to evaporated water), by acquiring more carbon in exchange for the water transpired by the crop (higher transpiration efficiency), or by partitioning more of the achieved biomass into the grain (higher harvest index). While the ratio of transpired to evaporated water is mainly under the dependence on crop and water management, transpiration efficiency and harvest index variations are mainly driven by genetic factors (Gregory et al. 1997). Transpiration efficiency was shown to be negatively associated to Δ (Farquhar et al. 1982). In the present study, significant differences were found between Iranian landraces and the modern cultivar Zakros for harvest index and not for Δ, suggesting that the difference observed for WUE_G between the two types of germplasm was due to carbon partitioning rather than to transpiration efficiency differences. Similar results have been obtained by Khazaei et al. (2008) in a set of Iranian diploid, tetraploid and hexaploid wheats. The results of this study all together indicate that under well-watered conditions, Δ does not represent an accurate predictor of yield but can be used for estimating WUE. They also suggest that WUE_G under well-watered conditions has been improved by modern selection mainly through an increase of harvest index.

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Review

In vitro selection and regeneration methods for wheat improvement

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Abstract

Wheat is an important staple food crop of world. Work on improvement of wheat is being done since long back using various methods and techniques. Emerging the field of biotechnology especially plant tissue culture has revolutionized the entire scenario of crop improvement including wheat. Regeneration of plants from callus and *in vitro* selection is the most important strategies that have been used to obtain efficient regenerated lines and subsequently genetic manipulation of wheat using *in vitro* techniques. Despite the significance of the problem for the successful implementation of *in vitro* techniques, the factors which control the establishment and regeneration of callus, the relative importance and the role of explants source, media, growth regulators, genotype, somatic embryogenesis, *in vitro* selection and *in vitro* induced variations are being discussed in the present paper.

Introduction

Wheat is the dominant grain of world commerce and is the staple food of millions of people globally. It is often used to produce a large variety of foods that include many kinds and types of breads, cakes, noodles, crackers, breakfast foods, biscuits, cookies, and confectionary items. Wheat, after rice, is the second most important crop in India and the World. It is a staple food for one-third of the global population. The present wheat is believed to have originated in Euphrates Valley as early as 10,000BC, making it one of the world's oldest cereal crops. It is also believed that wheat developed from a type of wild grass native to the arid lands of Asia Minor. Initially, Schulz (1913) grouped all the species of wheat in three natural groups, einkorn, emmer and dinkel wheat. Further, Sakamura (1918) established the three natural groups of wheat form a polyploidy series with haploid chromosome number n = 7, 14 and 21, respectively. During their agricultural selection, modern wheat strains which are hexaploid, with six sets of chromosomes per cell, evolved from their wild diploid relative parallels, in terms of chromosomes. Einkorn wheat has 14 chromosomes, which are designated AA. This species is believed to have hybridized with a wild grass species to produce emmer wheat. Later it was observed that wheat has the AA chromosome sets and the BB sets of the wild grass. This tetraploid, AABB, now mated with yet another wild grass species, which had the chromosome complement designated DD, to yield AA BB DD, our modern hexaploid wheat, which has 42 chromosomes. An important application of polyploidy breeding has been the development of the wheat rye hybrid called *Triticale*. This resulted from an attempt to combine the high yield and high seed protein content of wheat (*Triticum*) with the adaptability to adverse environmental conditions (such as cold and drought) and high-lysine content of rye.

Wheat is fairly rich source of vitamins, niacin, thiamine, energy and mineral suitability of wheat for many food products depends on its unique protein. Elevated grain protein content (GPC) and its quality influences the bread making properties of wheat (Mesfin et al. 2000). Industrial uses of wheat include

Table 1. Wheat production of major wheat producer countries of the world in three consecutive years

Wheat production (Mt)	2004-05	2005-06	2006-07	% change
World Production	629	618	587	-5.0
China	92	98	103	5.0
EU25	136	123	116	-5.0
India	72	69	70	1.0
Russia	45	48	44	-0.8
United state	59	57	49	-14.0

production of starch, gluten, distilled sprits and malts, etc. Wheat bran is rich in protein (14-18%) and vitamins. It is also used to feed livestock. Wheat straw is used as feed, animal bedding, compost and for making corrugated paper.

The wheat grain contains starch (60-70%), protein (10-17%), fiber (2-2.5%), fat (1.5-2.0%), sugar (2-3%) and mineral matter (1.5-2.0%). Worldwide, wheat is cultivated in an area of about 232 million hectares with an annual production of about 640 million tonnes. Globally, for the last five years, total wheat production is on the decline and during 2006-07 only 587 million tonnes of wheat was produced (Table 1).

Wheat improvement through classical breeding approaches in the 20th century

Wheat improvement work on systematic lines commenced only during the first decade of 20th Century. The period between "1904-1962" can be divided into three distinct phases and is well documented by Pal (1966). During first phase, beginning from 1904, the pioneering work was done by Albert Howard and Gabriella Howard who utilized a system of selection from single plants which enabled them to produce several wheat varieties like Pusa 4, Pusa 6 and Pusa 12. These varieties were popular not only in India but also in South Africa, Rhodesia and Hungary (Howard and Howard 1910). The semi-dwarf wheat varieties developed in Mexico by Norman Borlaug and his colleagues using the Norin 10 dwarfing genes, *Rht-B1* and *Rht-D1* attracted the attention of Indian wheat breeders.

The low input traditional agriculture, traditional tall wheat, inadequate irrigation facilities and the overall stagnate rural economy kept wheat production at a very low level. Suddenly, the wheat growing areas of India was bifurcated between India and Pakistan in 1947. Up to 1965-66, breeding efforts did not result in substantial yield improvement due to lack of suitable genotypes which can respond to fertilizer and irrigation. India became dependent on large-scale imports to feed its growing population, a situation which was described as "ship to mouth". Then era of semi-dwarf wheat came, which became an instant success and contributed to the "wheat revolution". The new genotypes triggered off complementary package of technologies, services and public policies which gave birth to what was termed as "green revolution" by Willium Gaud of U.S.A. in 1968. India never looked back from this stage. Nagarajan et al. (1998) observed that small gain has been obtained in wheat after every 5-10 years. At the time of release of Kalayan Sona, the yield level was about 4.2 t/ha. The next jump was made by the release of varieties like WL 711 and Arjun (HD 2009) in 1975, which resulted in a marginal yield gain of five percent.

Current scenario of wheat production

World wheat production in 2006-07 is 31 million tonnes lower than in the previous season. Harvesting of northern hemisphere wheat crops is complete, while in the southern hemisphere, harvesting is approaching completion. Many of the world's key producing countries have lower production in 2006-07 than in 2005-06 (Table1). Combining 2006-07 production with beginning season stocks, global wheat supplies were 722 million tones, 34 million tonnes less than in 2005-06. The lower wheat supplies have been driving up world wheat prices (US hard red winter, fob Gulf ports), which have averaged above US$200 a tonne. The world wheat indicator price is forecast to increase by US$32 a tonne in 2006-07 to average US$208 a tonne.

The success story of wheat in India during the last 30-35 years is unparalleled in the history of any other country. The wheat production in India, which was just 6.4 million tones in 1950s, has progressively increased to 76.6 million tones in 2000. The average productivity has increased from 6.5 quintal per hectare to 27 quintal per hectare. The state average of Punjab and Haryana are close to 40 quintal per hectare. The more recent genotypes like PBW 299, UP 2338, UP 2425 and PBW 343 derived from further crosses the various genotypes have higher yield potential of up to 55-60 q/ha.

The post-green revolution breeding programme were mostly concentrated towards further improvement in yield, selection for adaptability to different agro climatic condition and improvement of disease resistance particularly for rusts. The strategy was to cross some of the Indian wheat varieties with the semi-dwarf wheat varieties from Mexico to combine the grain characters and Chapati making qualities of local wheat with the photo-insensitivity, disease resistance and input responsiveness to dwarf types.

Constraints in wheat productivity

Today the wheat production may look satisfactory but the fact remains that the sudden surge in population and the First World War saw India becoming a food grain deficit nation. In fact, today we suffer from overflow of go-down with stored grains. India needs a buffer stock of 14-16 million tones for food security, but today it has more than 50 million tones of grains in buffer stock. National food security has not ensured food and nutritional security for individuals. India has 25 percent of world population below poverty line who cannot get two square meals per day. The calorie intake per day is less than 1500. Main reasons for this dichotomy of famine within population are poor socio-economic condition clubbed with high cost of wheat production. If India has to avert mass scale hunger despite green revolution, it has to face challenge to ensure equitable distribution of food grains at an affordable price. The later can be achieved only by replacing varieties of green revolution that require costly input of energy, water, fertilizers and pesticides with varieties that were, besides being efficient in utilization of water and fertilizers, also resistant to biotic and abiotic stresses. To achieve this objective at accelerated pace; the conventional methods of breeding will need to be complemented with modern tools of biotechnology such as plant tissue culture and genetic engineering techniques.

Biotechnological approaches in wheat improvement

Earlier the production of wheat lines with improved quality characteristics relied on traditional plant breeding techniques which has a limitation with the complex transfer of multiple associated traits, relating to both agronomic and end-use quality attributes. Certain trait like the amino acid composition of storage proteins for nutritional quality improvement is difficult to alter through conventional breeding techniques or at least not without adverse effects on other quality traits. The advent of recombinant DNA technology led to the specific gene transfer methodology with a chance to alter a trait specifically while retaining the superior qualities of a known cultivar. The transgenic technology adopted for wheat improvement needs identification of desired gene conferring the said trait, optimal method of gene transfer, *in vitro* regeneration and selection.

For development of transgenic wheat, the regeneration capacity during *in vitro* culture has to be very efficient so that stable transgenic with useful gene(s) can be developed. Regeneration frequency of wheat during *in vitro* culture is a limiting factor for the creation of transgenic. Tissue culture methods using mature embryo culture followed by callusing/somatic embryogenesis and selection of callus for high regeneration capacity may speed up varietals development through rapid attainment of homozygosity. Because of the limitation of early generation testing for yield, wheat biotechnologists have been interested in ways that can cut down on the time taken to reach homozygosity so that emphasis can be placed on testing the homozygous lines rather than the efforts on producing them. Three approaches are (i) single seed descent (ii) selection of high regenerated calluses using mature/immature embryo culture and (iii) production of haploid plants either by wide crosses or anther culture, followed by chromosome doubling.

The applicability of biotechnology to crop improvement induces non-conventional plant breeding methodology. These new technologies will not replace the traditional breeding techniques but should mutually complement them by enhancing efficiency, trait transfer precision and recovery of useful, value added variation (Karp 1995). Useful variability can be generated without sexual recombination by *in vitro* selection/ variation (Larkin 1987).

***In vitro* regeneration and selection in wheat: present status**

Regeneration of plants from callus is central to most of the strategies that have been proposed to obtain efficient regenerated lines and subsequently used for the development of transgenic wheat with desired trait. Genetic manipulations of wheat using *in vitro* techniques have been performed by several coworkers. The various factors influencing the *in vitro* regeneration via callus are discussed below.

Explant source

Explants sources vary in their ability to generate variation (Skirvin et al. 1994). Highly differentiated tissue (root, leaves and stems) produce more variation than explants with pre-existing meristems (axillary's buds, shoot tips and leaf bases). Cultures give rise to normal or near-normal regenerated plants when no significant dedifferentiation (callus) step occurred (Peschke and Phillips 1992). Organogenesis can involve more than one cell. Stability differences in tissue culture originating from different explants sources often are caused by pre-existing variability in the explants source. More chlorophyll deficient variants are recovered from immature embryo cultures than from mature embryo cultures (Cai et al. 1990). The highest rate of callus induction (9.1%) and green plant production (0.8%) were obtained with wheat cultivar Apollo using anther culture (Konieczny et al. 2003).

Various explants sources, such as immature embryos, immature leaves, immature inflorescences, mature embryos, mesocotyls and apical meristems have been used for callus culture in wheat. Use of different explants sources in wheat have been given in Table 2.

Table 2. Different explants used for callus culture in wheat

Explants	Use/ Application	References
Immature embryo	Callusing, plant regeneration and somatic embryogenesis	Ahloowalia (1982); Arun *et al.* (1994); Bohorova *et al.* (1995); Subhadra *et al.* (1995); Lange *et al.,* (1998); Kothari *et al.* (1998); Ozgen *et al.* (1998); Jimeneze and Bangerth (2001); Khanna and Daggar (2001); El-Sherbeny *et al.* (2001); Zair *et al.* (2003); Przetokiewicz *et al.* (2003); Patnaik iet al. (2006)
Inflorescence	Callus development, somatic embryogenesis, plant regeneration	Vasil (1982); Taghvaii *et al.* (1998); He and Lazzeri (2001); Marcinska *et al.* (2001); Barro *et al.* (1999); Yadav and Chawla (2001)
Anther	Creation of genetic variation, callusing, regeneration	Moussa *et al.* (1999); Dogramaci *et al.* (2001); Holme *et al.* (1999); Redha *et al.* (1998); Konieczny *et al., 2003*.
Spikes	Grain growth	Arora and Singh (1998); Leon *et al*. (2006)
Microspore	Callus formation, Somatic Embryogenesis, Plant regeneration	Indirianto *et al.* (1999); Kunz *et al.* (2000); Anapiiaev (2000); Zheng *et al.* (2002); Liu *et al.* (2002)
Scutellum	Somatic Embryogenesis, Plant regeneration	He and Lazzeri (2001); Barro *et al.* (1999); Leon *et al.* (2006)
Root	Callus formation, Regeneration	Cai *et al.* (1999)
Seed	Callusing, regeneration, rooting	Karaki and Abu (1999)
Mature embryo	Callusing, Somatic Embryogenesis, Organogenic callus, regeneration	Ozias-Akins and Vasil (1983); Kato *et al.* (1991); Kosulina (1995); Racz *et al.* (1993); Dzhos and Kalshnikova (1998); Ozgen *et al.* (1998); Varshney *et al.* (1999); Sayar *et al.* (1999), El-Bahr *et al.* (2000); Mendoza and Kaeppler (2002); Delporte *et al.* (2001); Yadav *et al.* (2000); Nayal *et al.* (2002), Wang (2004); Turhan and Baser (2004); Zale *et al* (2004); Chen *et al.* (2006); Bi *et al.*(2007); Yao *et al.*(2007)
Unpollinated ovary	Green haploid plants	Sibi *et al.* (2001)

Callus culture

The isolation and successful establishment of callus cultures depends on several factors like explants source and culture conditions. Callus, which shows stable characteristics under specific conditions after sub-culture through many successive passages, is a suitable material for cytodifferentiation. The advantage of using such callus is that it is composed of a fairly homogenous mass of cells and can be proliferated in large amounts under known culture conditions.

Effect of media composition and growth regulators on wheat callus

Callus is initiated *in vitro* on cut or exposed cell surfaces in contact with a growth medium. Callus proliferation is a wound response. Excision of the explants stimulates the wound response *in vivo* (induction of stress-induced enzymes and other chemical by-products, and activation of transposable elements) which can be enhanced by growth regulators (McClintock 1984). Most plant growth regulators and specifically 2, 4-dichlorophenoxy-acetic acid (2, 4-D) and 6-benzylaminopurine have been implicated in tissue culture-induced variability (Evans 1988; Shoemaker et al. 1991). Racz et al. (1993) studied the effect of sucrose, 2, 4-D and different vitamins on callus induction in winter wheat mature embryo explants. Solid MS medium was optimum for mature embryo culture (Leon et al. 1995;

Dzhas and Kalashnikova 1998; Varshney et al. 1999; Sayar et al. 1999; Mendoza and Kaeppler 2002; El-Bahr et al. 2000; Delporte et al. 2001) of wheat supplemented with different combinations of plant growth regulators. Immature embryo culture supplement with 2, 4-D (Subhadra et al. 1995; Taghvaii et al. 1998; Rao and Chawla 1998; Kothari et al. 1998; El-Sherbeny et al. 2001; Shan et al. 2000) gave good callus growth. The effect of B5 (Rao and Chawla 1998), LS and potato medium (P1, P2 and P4) (Chaghamirza and Arzani 1999) were tested for callus induction. Immature embryos were cultured in liquid MS medium supplemented with 2 mg l^{-1} 2, 4-D to obtain variation (Ahmed and Sagi 1993).

Immature embryos of 1.0-1.5 mm (14 d after anthesis were cultured on MS medium supplemented with 1.5 or 2.0 mg l^{-1} 2, 4-D and found that 90-100% of these embryos formed callus (Arun et al. 1994). The callus induction frequency ranged from 51 to 81% and compact, nodular and morphogenic callus frequency ranged from 35 to 50%. The medium × cultivar × 2, 4-D level interaction were significant (Rao and Chawla 1998). Addition of 2, 4-D at 3 mg l^{-1} in combination with zeatin at 0.5 mg l^{-1} was beneficial for the formation of morphogenic callus (Dzhas and Kalashnikova 1988).

Ozgen et al. (1998) compared the responses of mature and immature embryo cultures. Immature embryos were aseptically dissected from seeds and placed with the scutellum upward on a solid agar medium containing the inorganic components of Murashige and Skoog (MS) medium and 2 mg l^{-1} 2, 4-D. Mature embryos were placed furrow downwards in dishes containing 8 mg l^{-1} 2, 4-D for callus induction. The immature embryos formed the highest amount of callus on MS media supplemented with 2, 4-D and Kinetin while mature embryos formed the highest amount of callus with 2,4 D. Anthers of a wheat F_1 hybrid (Alondrax sumai 3) on W14 media with different concentrations of salicylic acid (SA) and epi-brassinolide (epi-BR) were cultures and observed the significant differentiation and increased rates of callus development (Yang et al. 1999). Cai et al. (1999) obtained calluses of wheat from seminal roots grown in media containing 2-6 mg l^{-1} 2, 4-D. Anthers from a doubled haploid line of spring wheat cv. Pavon 76, plated in liquid P-4 medium supplemented with 2.0 or 4.0 mg l^{-1} 2, 4-D developed good calluses (Zheng and Konzak 1999). Immature embryos of two wheat genotypes were used to establish callus cultures (Jimenez and Bangerth 2001) for the study of endogenous effects of growth regulators (Marcinska et al. 2001). Calluses were induced on MS medium with 1-2 mg l^{-1}, 2, 4-D and 1.5 mg l^{-1} BA using hypocotyls segment (Jin et al. 2002).

The primary event causing tissue culture induced variability may be cell cycle disturbance caused by exogenous hormone (Peschke and Phillips 1992) or nucleotide pool imbalances (Jacky et al. 1983). Sister chromatid exchange frequency can increase with a low concentration of 2, 4-D (Dolezel et al. 1987).

Somatic embryogenesis in wheat

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. According to Sharp et al. (1982), somatic embryogenesis is initiated either by pre-embryogenesis determined cells (PEDCs) or by induced embryogenic determined cells (IEDCs). The embryogenesis process can be divided into two main and fundamental phases: a first 'morphogenetic' stage where the embryo proper is formed and organs and tissues of the future plant are specified and a second 'metabolic' stage characterized by the storage of reserves (carbohydrates, proteins, lipids) in preparation for germination. Excellent reviews are available which focused on zygotic and somatic embryogenesis and comparisons between them (Goldberg et al. 1989; Wilde et al. 1995; Laux and Jurgens 1997; Dodeman et al. 1997; Rojas-Herrera et al. 2002). Exposure of the suspension cultures to high concentrations of 2, 4-D produces clusters of small isodiametric cells, known as pro-embryogenic mass (PEMs) (Halperin 1966). The subsequent elimination of auxins from the culture medium allows the expression of embryogenesis. One of the basic components of a medium influencing somatic embryogenesis of cereals is the type of auxin (Przetakiewicz et al. 2003). It is now accepted that, in the continuous presence of auxins, proembryogenic masses synthesize the gene products required to complete the stage of embryogenesis as well as the mRNA that can inhibit the somatic program. The depletion of auxins will result in the inactivation of a certain number of genes, permitting the embryogentic program to proceed; the transition to further developmental stages may require new gene products (Zimmerman 1993). It has been observed that the exposure of cell cultures to high concentrations of auxins can provoke hypermethylation of DNA (Lo -Schiavo et al. 1989) which, in turn, may cause transcriptional inactivation of methylated genes (Carman 1990). In the recent past, considerable success was achieved in obtaining reproducible regeneration of plants from embryogenic cultures of all major cereals.

Arun et al. (1994) reported that about 53-91% of the calluses produced somatic embryos and the frequency of embryogenesis depends mainly on the cultivar. Subhadra et al. (1995) obtained SEs from cell suspension culture of wheat. Marciniak et al. (1997) showed the effect of media and genotypes by anther culture on embryoid induction frequency (from 2.4 to 22.5 per 100 anthers). Barro et al. (1999) developed media (supplemented with picloram) for somatic

embryogenesis from immature inflorescences and immature scutellum of elite cultivars of wheat and barley. Kunz et al. (2000) standardized an efficient protocol for the production of embryos in isolated wheat microspore culture. The scutellum cultures gave higher frequency of embryogenesis as compared to inflorescence culture (He and Lazzeri 2001). The effect of ovary-conditioned medium on microspore embryogenesis in wheat was well documented by Zheng et al. (2002). Ahmed et al. (2002) obtained somatic embryos from shoot apical meristem of wheat on media containing BA and 2, 4-D (0.5 mg L^{-1}) with high frequencies of embryogenic calluses (48.6%) from immature embryos of wheat (El-Sherbeny et al. 2001) and 47% embryogenic callus induction rate using fragmented mature embryo cultured on MS media supplemented with 5M 2, 4-D (Delporte et al. 2001). Secondary somatic embryos were produced from somatic embryos which attained somatic clonal capability and proved beneficial for improving the transgenic system and promoting the genetic transgenic efficiency in wheat (Wu et al. 2005).

***In vitro* selection in wheat**

The frequency of total somaclonal variation should be high enough for selection of derivable traits. The *in vitro* × genotype × explant source interactions contribute to one level of variation. When *in vitro* selective agents are added to the protocol, additional interactions are encountered (Handro 1981).

Selection in culture

A major challenge for exploitation of somaclonal variation is the identification of useful variation (Evans 1988; Smith et al. 1993). Because cells are genetically variable in culture, specific traits can be selected using *in vitro* induced spontaneous mutations. Cells may be selected in either a positive or negative manner using direct (cells cultured with selective agents followed by isolation of surviving cells), rescue (cell exposure to culture conditions, capable of killing or inhibiting susceptible cells followed by culture under different conditions to recover survivors) and gradual increase in selection pressure over time (Conner and Meredith 1989). *In vitro* selective agents have been used to detect useful variants, for example, NaCl (Arzani and Mirodjagh 1999; Zair et al. 2003; Yadav et al. 2004) for salt tolerance, $AlCl_3$ for the Aluminum toxicity portion of acid soil tolerance (Conner and Meredith 1985; Bamabas et al. 2000), grain polyphenol variants (Cai et al. 1995), polyethylene glycol (Adkins et al. 1995) as an osmo-regulator to stimulate drought stress, *in vitro* regeneration of wheat under different stress of mannitol , mannose (Yao et al. 2007), *in vitro* screening of scab resistant wheat lines tolerant to deoxynivalenol (vomitoxin) (Lu et al. 1998); ABA for freezing tolerance (Sapina et al. 1994).

In vitro selection technique has been used to improve cold hardiness (Galiba 1994); drought tolerance (Gawande et al. 2006; Bajji et al. 2004), Salt tolerance improvement (Zair et al. 2003; Yadav et al. 2004), disease resistance (Svabova and Lebeda 2005); herbicide resistance can also evolve from somatic cell selection (Saunders et al. 1992); for resistance against *Helminthosporium sativum* in wheat and barley (Chawla and Wenzel 1987); and fusaric acid resistant barley plants . *In vitro* induced selection for high frequency regeneration (Yadav et al. 2000; Ye et al. 1998; Machii et al. 1998; Cai et al. 1999) in wheat; incorporation of useful agronomic traits in rice (Jain 2001) agronomic performance and quality characteristics of tissue culture derived lines of wheat (Villareal et al. 1999) and for increase the gliadin and glutenin subunits and protein content in seed of progenies from regenerated plants of wheat has been successfully implemented (Hu et al. 1998). Shi et al. (1998) developed six new wheat lines for resistance to powdery mildew through *in vitro* culture selection

***In vitro* regeneration in wheat**

There are only a few reports on plant regeneration from cultured mature embryos of wheat. Early reports on regeneration showed a low frequency (7% of one culture) of plant regeneration (Chin and Scott 1977). Twenty percent regeneration was obtained through the use of NAA (1 mg l^{-1}) plus kinetin (5 mg l^{-1}) in the regeneration medium. In general a downshift in auxin concentration results in organogenesis and plant regeneration (root and shoot induction) after transfer of the callus either on a medium containing certain combination of plant growth regulators or no regulator at all (Shimada et al. 1969; Sharma et al. 1981; Bajaj 1986; Ovesna and Lhotovia 1987; Elena and Ginzo 1988; Kintzios et al. 1996; Delporte et al. 2001).

Eapan and Rao (1982) observed increased plant regeneration rates from wheat mature embryo explants cultured on media supplemented with IAA and cytokinin, mostly zeatin. A low exogenous auxin application (such as NAA at 1 mg l^{-1}) initiates only roots (Chin and Scott 1977; Ahloowalia 1982).

Racz et al. (1993) used mature embryos of winter wheat cultured in MS-B medium supplemented with $AgNO_3$ (20 mg l^{-1}) and found enhanced regeneration. Leon et al. (1995) studied the effect of various media constituents (like ammonium, calcium, sucrose) and different medium (Taghvaii et al. 1998) on regeneration of mature embryo culture.

Varshney et al. (1999) reported regeneration frequency as high as 94% on the regeneration medium containing NAA (0.2 mg l^{-1}) plus BAP (1 mg l^{-1}) in 17 cultivars of *T. aestivum* and 3 cultivars of *T. durum* through mature embryo culture. Wang et al. (2002) achieved 70% plant regeneration frequency through embryogenic calluses of wild rye using mature embryos. El-Bahr et al. (2000) cultured mature embryos in MS media with 2, 4-D with different

concentrations of NaCl for 12 weeks to select the salt tolerant wheat lines. Mendoza and Kaeppler (2002) used 2, 4-D and dicamba to enhance the number of regenerated plants per embryo and reduce the amount of time required for plant regeneration by 3-4 weeks. Some new techniques such as the endosperm supported callus induction method have been successfully used in callus induction from mature embryos (Bartok and Sagi 1990; Ahmed et al. 1992; Ozgen et al. 1996; Sayar et al. 1999). Nayal et al. (2002) obtained regeneration in wheat var. Sonalika using Kinetin (0.5 - 1.5 mg l^{-1}) with IAA (0.5 mgl^{-1}) in MS media. Yadav et al. (2000) selected R_1 lines from wheat genotype UP2338 which gave a maximum of 19 shoots from a single callus in the MS medium supplemented with IAA and kinetin (Fig. 1 A-D).

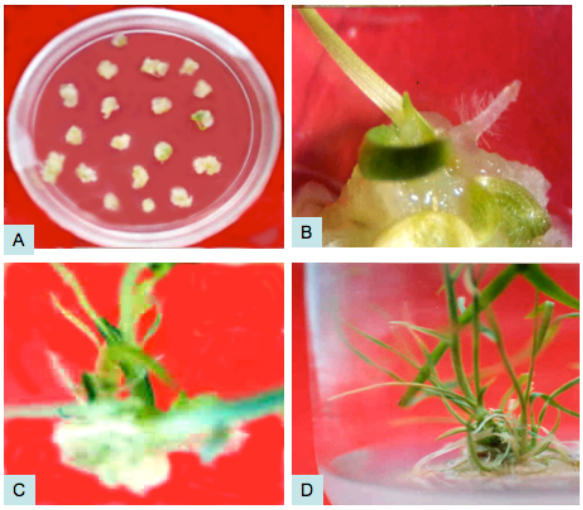

Fig. 1. (A) Callusing in Indian wheat genotypesof *in vitro* selected lines using mature embryo cultures (B) Initiation of shoot and root primodia (C) Multiple shoot induction and elongation (D) Multiple shoot regeneration of in vitro selected lines

Factors influencing *in vitro* regeneration potential of different explants of wheat presoaking time

The length of the imbibition period could cover different phases in the release from inactivity of the embryo, rehydration of the tissues, hormonal activation, transformation of reserves etc and might therefore influence the reactivity of the explants in culture. Chlyah et al. (1990) used several durations (16 to 64 h of imbibitions) and reported that with 20 h soaking time of mature embryos, nearly 100% of explants formed somatic embryos.

Effect of genotype

The importance of genotype in determining the *in vitro* response of wheat tissues has been recognized and the efficiency of callus induction, callus growth rate and plant regeneration frequency have all been reported to be genotype dependent (Zhou and Lee 1983; Racz et al. 1993; Ozgen et al. 1998; Yadav et al. 2000; Anapiiaev 2000; Schween and Schwenkel 2003; Yadav and Chawla 2001; El-Sherbeny et al., 2001). Varshney et al. (1999) evaluated 17 cultivars for their ability to produce embryogenic callus from mature embryos and reported that percentage regeneration varied widely from cultivar to cultivar.

Fragmentation, orientation and size of embryos

Chlyah et al. (1990) attempted to determine the most embryogenic zones of the wheat embryo by fragmenting it in various ways prior to culture. It was observed that fragmentation of the embryo could bring about the formation of as many embyogenic calli as explants and a large number of cells in different parts of the embryos became capable of initiating embryogenic callus by fragmented original mature embryos (Delporte et al. 2001).

Eapen and Rao (1982) studied the effect of orientation of embryos on callus induction. The embryos were placed on the medium in three different ways: (a) scutellum facing up (b) scutellum in contact with the medium and (c) one side of scutellum and embryonic axis in contact with the medium. Frequency and intensity of callus development were found to be best in orientation (a) and the least in orientation (c). Chang et al. (2003) showed the effect of embryo size for the establishment of embryogenic callus in barley cultivar (*Hordeum vulgare* L cv. Morex). Small size embryo was found excellent for embryogenesis and regeneration.

Other physiological factors

Recent studies suggest that genotype determined ratios of endogenous growth regulators were responsible for rapid *in vitro* regeneration (Marcinska et al. 2001; Jimenez and Benegerth 2001). The influence of genotype and cold pre-treatment was well documented on production of embyoids and regeneration of wheat (Mentewab and Sarrafi 1997; Xynias et al. 2001).

Effect of low temperature treatment (Hau and Teng 1994) and combined effect of colchicine, L-proline and post inoculation low temperature (Redha et al. 1998) was associated with fast differentiation rate in wheat. The effect of low intensity laser radiation (632.8 nm, 12 mW) on morphogenetic and regeneration processes in wheat (cv. Skala) callus culture were investigated by Salyaev et al. (2001). They observed a marked increase in the number of regenerated plants when the callus was subjected to irradiation. The final yield of regenerated plants comprised 38% in irradiated samples vs. 25% in controls ones.

Lange et al. (1998) attempted to study the genetics of *in vitro* organogenesis and precocious germination of wheat embryos. The results indicated a complex gene action controlling both traits, with additive, dominance and epistatic effects. High broad-sense heritability values were found, indicating genetic

determination. In view of the complex gene control of these traits, it is suggested that genetic improvements can be achieved by selecting for the traits in advanced generations of the segregating population.

Occurrence of somatic variation

Somatic variation refers to all variability observed among tissue culture regenerated plants (Larkin and Scowcroft 1981). Tissue culture regenerated variants have also been called calliclones (Skirvin and Janick 1976), phenovariants (Sibi 1976), protoclones (Shepard et al. 1980) and subclones (Cassells et al. 1991). Chromosome multiplication and structural observations induced during culture may constitute the basis of gametoclonal and somaclonal variations (Dogramaci et al. 2001). *In vitro* culture *per se* can be extremely stressful on plant cells and involve highly mutagenic processes during explants establishment or callus induction, maintenance, embryo induction and plant regeneration (Lorz et al. 1988). Two types of somaclonal variation can result: epigenetic (developmental) and heritable variation (Skirvin et al. 1994).

Epigenetic variation can be transient or temporary in later generations even when the material is sexually propagated. This variation includes phenotypic changes that involve expression of specific genes (Hartman and Kester 1983). Because explants adapt to an *in vitro* environment in step wise fashion by becoming more juvenile, the resulting calluses may vary in maturity from juvenile to fully mature. Plants regenerated from these tissues also vary depending on the developmental stage progression of the tissue when the stimulus to regenerate is applied (Skirvin et al. 1994). Shoot regeneration from dedifferentiated callus can produce an immature, unstable clone that may eventually revert to the original parental clone. Broad spectrum variation is available through either nuclear or cytoplasmic sources; all types of variation could potentially be recovered and used for crop improvement (Elkonin et al. 1994; Skirvin et al. 1994; Khanna and Garg 1997). Heritable variation is stable through the sexual cycle and with repeated asexual propagation (Skirvin et al. 1994). Stable genetic alterations involve phenotypic and biochemical traits due to karyological variations (gene or chromosome aberrations) (Lorz et al. 1988). Heritable somaclonal variation involves either single or multiple gene changes, including alterations in DNA bases, chromosome or the entire genome (Orton 1994), single base pair changes, paracentric and pericentric inversions, deletions, translocations and ploidy changes (D'Amato 1991; Garcia et al. 1994). Cytoplasmic variation has been found, including mitochondrial controlled male sterility (Elkonin et al. 1994). Chimeral plants subjected to tissue culture can produce a high percentage of variants (Skirvin and Janick 1976; Skirvin et al. 1994). Chimeric regenerant frequencies range from 10-70% in sorghum and 54-79% in maize (Cai et al. 1990). However, chimeric regenerants in some plant species are rarely observed (Vasil 1983). Variation may represent pre-existing variation or variation induced during callus formation and not during the shoot formation process (Skirvin et al. 1994).

Culture age enhances variability of regenerated plants (Symillides et al. 1995; Kothari et al. 1998). This age effect can be attributed to (i) increased mutation rate per cell generation (Murashige and Nakano 1965) and accumulation of mutations over time (ii) a lag period of apparent culture stability caused by early generation mutations that are not detected until sufficient mutant cells have accumulated (Benzoin and Phillips 1988), (iii) active selection of early culture mutations that increase in number over time (Amstrong and Phllips, 1988) or (iv) increased ploidy (Colijin-Hooymans et al. 1994). As callus induction time increases, the morphogenesis potential decreases, whereas the frequency of albino shoots and callus producing only roots increases (Wen et al. 1991). Some mutations may occur in sequence throughout the callus phase rather than occurring randomly or all together at an early culture stage (Fukui 1983).

Regeneration competence in *Gramineae* may be rapidly lost during differentiation and senescence (Ozias-Akins and Vasil 1988). This loss may be due to the endogenous concentrations of growth regulators (Vasil 1987). Tissue culture variability rate can increase as growth regulator concentration increases (Skirvin et al. 1994).

In vitro genetic control of regeneration could be functioning specifically on response to plant growth regulators (Komamine et al. 1990). Genes controlling phytohormone signals are directly involved in plant regeneration competence (Henry et al. 1994), negating the concept that 'dominant' genes govern regeneration and that all cultivars have genes for regeneration. Heritability ranges of 9 to 60% (embryo germination); 30-55% (callus induction) and 15 to 49% (embryogenic callus formation) have been documented in wheat (Chevrier et al. 1990). Recently, some somaclonal variants were screened for resistance to scab (Yang et al. 1998; Liu et al. 1997), improvement of agronomic traits, salt and drought tolerance in wheat (Villareal et al. 1999), Somaclones (R3 and R4 generations) regenerated from five winter wheat genotypes were evaluated for variation in five agronomic and morphological characters (Ivanov et al. 1998).

Field selection of variants

Haploidization, somatic embryogenesis, cell suspension culture and protoplast technology all require plant regeneration techniques for genetic manipulation and subsequent selection in plant improving programs (Henry et al. 1994). The entire *in vitro* process from genotype and explants selection to

media induced callus, embryo and plantlet regeneration is extremely productive in creating variation (Hossain et al. 2003). Somaclonal variations for a wide range of agronomically important traits and yield have been observed in sorghum, sugarcane, maize and wheat etc. In case of wheat somaclonal variations for grain colour, height, and tillers per plant and seed storage proteins were reported by Ahloowalia (1982). Nevertheless, somaclonal variation has not been incorporated as a standard protocol in most plant breeding selection schemes. Perhaps this limited integration can be traced to the segregation of biotechnology and traditional breeding laboratories or the limited understanding of molecular events that trigger *in vitro* induced variation coupled with strong beliefs in traditional breeding methodology.

Phenotypic variation

Phenotypic variation in somaclones resulting from emrbyogenic callus regeneration is increased with increasing time in the callus and regeneration phases (Cai et al. 1990). Phenotypically similar variations can segregate in progeny of different callus lines or reappear in later generations. Positive variations in height, maturity, biomass, grain yield, tillering, ploidy level and kernel oil content (Cai et al. 1990; Encheva 1993; Bhaskaran et al. 1987) have been documented from various *in vitro* programs.

Field screening techniques

A major failure for the release of more useful regenerants may be due to inappropriate germplasm/segregating progeny selection techniques or improper field screening/evaluation methods. Many of the field tests that have evaluated somaclonal variants have been conducted with small, unreplicated trials because of low seed availability (Mitchell et al. 1992; Maddock 1985). Inadequate or negative phenotypic/genetic variation between regenerants and their donor parents has discouraged wide scale adoption into many *in vitro* selection and breeding programs (Baille et al. 1992). It is felt that agronomic performance of somaclonal variants lines need to be confirmed in relevant field tests before they are incorporated in production programs (Arnoldo et al. 1992; Encheva et al. 2003).

Conclusion and future prospects

There is a need for novel and innovative approaches for increasing the crop productivity involving traditional plant breeding and modern biotechnological approaches. In wheat there is a need for developing superior genetic stocks with high quality grain, high fertile tillering, and more spikelets/spike using *in vitro* regeneration and selection methods.

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Wheat Inf. Serv. 106: 29, 2008. www.shigen.nig.ac.jp/ewis

Others

Editorial

Shigeo Takumi's two-year term as Editor in Chief ended in March 2008. Shigeo did a great job in launching eWIS. During his time, eWIS safely navigated the transition into the age of electronic publishing. We all thank him for his herculean effort. For the next two years, Yoshi Matsuoka will serve as Editor in Chief. Tsuneo Sasanuma will serve as Vice Editor in this term.

The Editorial Office is running smoothly. The Office is now ready for more work, especially for international submissions. In the period from February 2006 to February 2008, eWIS published 15 Research Information papers. This figure may not seem too bad, but it means that, on average, there are only three Research Information papers per issue. Accordingly, our mission in the term is to make a take-off from the situation. WIS is now listed in e-journals (http://www.e-journals.org/botany/) and we hope that this will make our newsletter more accessible.

This year, Dr. Xueyong Zhang (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China) joined the Advisory Board. We are grateful for his understanding and look forward to his support during the term.

Lastly, but not the least, we would like to thank Shingo Sakaniwa and Takehiro Yamakawa (Yamazaki lab, National Institute of Genetics, Japan) for their enduring technical support.

September, 2008

Editorial Office
Yoshihiro Matsuoka
Tsuneo Sasanuma
Goro Ishikawa
Shuhei Nasuda
Shigeo Takumi

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References should be cited in the text by the author(s) and year, and listed at the end of the text with the names of authors arranged alphabetically. When an article has more than two authors, only the first author's name should appear, followed by "et al.", in the text. The references should be formatted as follows.

Journal articles:

Payne PI, Holt LM, Law CN (1981) Structural and genetical studies on the high molecular weight subunits of wheat glutenin. Theor Appl Genet 60:229-236.

Book chapters:

Peacock WJ, Dennis ES, Gerlach WJ (1981) Molecular aspects of wheat evolution: repeated DNA sequences. In: Evans LT and Peacock WJ (eds.) Wheat Science - Today and Tomorrow. Cambridge Univ. Press, Cambridge, UK, pp. 41-60.

Books:

Knott DR (1989) The Wheat Rusts - Breeding for Rust Resistance. Springer-Verlag, New York, USA.

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communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., K. Tsunewaki personal communication).

Abbreviations
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Nomenclature
Nomenclature of genes and chromosomes should follow the 'Catalogue of gene symbols for wheat' (McIntosh *et al.*: 10th Int. Wheat Genet. Symp. 2003).

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The DDBJ/EMBL/GenBank accession numbers must be provided for newly reported nucleotide sequences.

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