

Discovery of desirable genes in the germplasm pool of *Aegilops tauschii* Coss.

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Abstract

In the present study, a set of 63 accessions of *Aegilops tauschii*, the D-genome donor of bread wheat, was evaluated for marker-trait association using SSR markers and biotic and abiotic stress tolerance. Five accessions of *Ae. tauschii* (TA1644, TA1642, TA1695, TA2452 and TA2473) were resistant to Hessian fly whereas seven (TA1649, TA2460, TA2450, TA2541, TA2470, TA2397 and TA749) were resistant to leaf rust pathogen. However one accession TA1695 was also resistant to pest green bug. Significant associations across D-genome revealed that chromosome 2D is associated with resistance to several stress factors. Two alleles at *Xgwm296* locus were associated with resistance to leaf rust, Septoria leaf blotch, green bug, and salt tolerance. Another locus *Xgwm261* detected association for resistance to leaf rust, Septoria leaf blotch and Hessian fly. The existence of a QTL for tolerance to high salt concentration was also detected through association with marker *Xcfd11*, located on this chromosome. Furthermore association analysis also detected two SSR markers, *Xgwm261* and *Xgdm35*, each with a fragment amplified in the leaf rust resistant accessions while lacking in the susceptible accessions. This study reveals the potential of association analysis in identifying the genomic regions having strong influence on useful traits. Through association mapping the germplasm collections can be initially screened and characterized to identify candidate genetic stocks and genomic regions. Subsequently, bi-parental crosses can be initiated for fine genetic mapping of genes or QTLs through linkage analysis.

Key words: Marker-trait association, SSR markers, biotic and abiotic stresses

Introduction

A gradually declining rate of genetic improvement in

yield potential of crop plants is the reflection of their narrow genetic base which needs to be reversed through rigorous use of wide germplasm in breeding programs. The wild relatives of crop plants have been reported to provide unique genes for enhancement of yield in rice [1], sorghum [2], oats [3] and fruit size in tomato [4]. Unfortunately the phenotypes of wild relatives of cultivated plants are a poor indicator of potentially useful genes present in them especially for agronomically important quantitative characters like yield, quality, and adaptation. Hence, special techniques of discovery and exploitation of novel gene complexes from wild species in the form of advanced backcross QTL analysis has been proposed by Tanksley and Nelson [5]. But equally important and challenging is the identification of desirable genes available in existing germplasm pools being maintained as land races, obsolete varieties and elite genetic stocks. Though genetic and geographic diversity of such stocks is the key guiding factor for selecting potential donors of new genes for crop improvement, even then the number of accessions so identified is too large to be accommodated in routine breeding programs. A number of attempts have thus been made to characterize genetic diversity with the help of easily observable molecular markers for precise identification and utilization of exotic germplasm through marker assisted selection. RAPD markers associated with five quantitative traits in rice have been reported [6]. These markers could reliably predict the performance of other samples of germplasm for these characters. Such an association of molecular markers with quantitative traits has also been reported in other crops [7-9].

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Recently association mapping approach has been used in different plant species to identify markers and genes associated with a variety of phenotypes. Marker-trait associations in wheat have already been worked out for a number of traits including high molecular weight glutenin [10], late maturity α -amylase activity [11], milling quality, seed size/seed shape [12], and resistance to *Stagonospora nodorum* blotch [13], leaf rust [14], and *Fusarium* head blight [15]. A number of major genes for resistance to diseases and insect pests are known in *Ae. tauschii*. A saturated molecular map and a collection of wide germplasm representing all major geographic areas of diversity are also available. The present study thus reports the scope and present status of gametic phase linkage disequilibrium (GLD) based association analysis to discover useful genes from germplasm pools of Tausch's goat grass *Ae. tauschii* Coss.

Materials and methods

Germplasm

A set comprising 63 genetic stocks from an initial collection of 546 accessions of *Ae. tauschii* being maintained by the Wheat Genetic Resource Center, Kansas State University, Manhattan was selected on the basis of their divergent reaction to diseases, insects, and salt tolerance. For this study, the accessions were evaluated for resistance to leaf rust, *Septoria tritici* blotch, tan spot, green bug, Hessian fly, nematode infestation, and high salt concentration. The selected accessions represented all the sub-species of *Ae. tauschii* i.e. *typica*, *anathera*, *strangulata* and *meyrii*, collected from different geographic regions including Iran (21 entries), Afghanistan (14), Azerbaijan (7), Turkey (3), erstwhile USSR (2), Pakistan (3), Japan (3), China (1), Armenia (4), Georgia (3), and Turkmenistan (2). Fifteen germplasm stocks of hard winter wheat, *T. aestivum*, with known genes for resistance to diseases and Hessian fly introgressed from *Ae. tauschii* along with recipient parental lines were also included in the study to verify the association of markers and genes through a comparison of marker profile of donor accessions with corresponding germplasm stocks.

Trait evaluation

The seedlings of all the accessions were evaluated for reaction of leaf rust (*Puccinia triticina* Eriks.) using the system of Browder and Young [16]. A line was considered resistant if associated with an infection type with a sporulation rating of 0, 1 or 2 on a scale of 0-9. The accessions were evaluated for reaction to *Septoria tritici* blotch (*Mycosphaerella graminicola* (Fuckel) J.

Schröt. in Cohn). The data were recorded in terms of different categories of 0-10, based on percentage of leaf area infected. The accessions with score of 1 or less were categorized as resistant. For tan spot screening the seedlings were inoculated with conidia of *Pyrenophora tritici-repentis* (Died) Drechs., by the methods of Riaz *et al.* [17]. Tan spot symptoms were recorded on 1-5 scale and accessions with score of 1 were categorized as resistant. The method described by Thomas and Conner [18] was used to determine the reaction of accessions to wheat curl mite. The accessions were classified as resistant or susceptible based on curling and trapping of the leaves. The reaction to Hessian fly (*Mayetiola destructor* Say) was determined by the methods of Cartwright and Lahue [19]. The plant reaction was determined about fifteen days after infestation and individual plants were classified as resistant or susceptible. Susceptible plants were stunted and dark green. Resistant plants were not stunted; they were yellowish green and showed a high level of antibiosis in that all larvae died in the first instar. For reaction to green bug (*Schizaphis graminum* Rond), ten apterous adult green bugs were placed on each plant at 2-3 leaf stage as per the method described by Harvey *et al.* [20]. Susceptible plants began to show generalized chlorosis after 5-7 days and were easily distinguished from dark green resistant plants. The Na and K concentration in most expanded leaves of each accession was measured from 26 days old plants which had been growing in 150 Mol⁻³ NaCl for 16 days.

The accessions were selected as to ensure representation of all geographic regions of diversity and comparable number of entries in resistant and susceptible categories for each of the above mentioned diseases and insects. The reaction with respect to nematode resistance had been recorded as number of white females per plant, which ranged from 0.8 to 50.0. Similarly for salt tolerance (Na⁺ and K⁺) also the magnitude of salt concentration in leaves was measured on a continuous scale. For these three characters the stocks were taken to represent entire range of variation in the collection.

DNA extraction and PCR amplification

Total genomic DNA was isolated from seedlings of 63 accessions of *Ae. tauschii* and fifteen germplasm lines of *T. aestivum* with known genes for resistance. The molecular profiling was conducted with thirty-five microsatellite primers specific to D genome and covering all the seven D-genome wheat chromosomes [21]. The polymerase chain reaction assays were carried out in

25ul reactions as described by Röder *et al.* [21] in a MJ Research thermocycler [Watertwon, MA, USA]. Products were separated on 2.3% Meta Phor agarose gels (FMC Bioproducts) in 0.5 X Tris-borate buffer. Gels were stained with ethidium bromide and visualized with UV light.

Data analysis

The sequence amplified by each primer pair was considered as a locus with each variant fragment as an allele. The presence or absence of each fragment was coded as 1 and 0 respectively to generate a binary data matrix. The association between the phenotypic performance and remaining 102 polymorphic fragments was tested by chi-square test with the Q-Gene software [22]. Fisher's exact test was performed for marker-character combinations where any of the cells in 2x2-contingency table had a count of less than five units. Single marker analysis for metric traits was also performed with Q-Gene to detect association of markers with resistance to nematode and salt concentration. The extent of association among microsatellite markers themselves was computed through chi-square test of all possible, 5151, pairs of markers through Q-Gene by coding the markers in the form of categorical data. The Type I error rate from the above analyses was adjusted by division of the probability values with the number (102) of simultaneous tests.

Results and discussion

Unlike for human populations the linkage analysis can easily be performed in plant populations to identify molecular markers linked to the genes for their use in marker-assisted selection. But here again the requirement of mapping population makes linkage analysis difficult and inappropriate for molecular tagging of all desirable genes available in large germplasm pools. The concept of GLD based association analysis in germplasm has been exploited to establish linkage of desirable genes with molecular markers for use in crop improvement. Some of the applications in rice [6]; barley [7, 8], and oats [9] suggest that a part of the observed trait-marker associations in germplasm pools also are created by physical linkage of corresponding genes and markers.

SSR marker polymorphism and its association with phenotypic performance

The total number of alleles at 35 SSR loci and chromosomal location of these markers is given in Table 1. The number of alleles ranged from 2-6 with an

Table 1. List of markers, their chromosomal location and number of alleles generated

S.No.	Marker	Alleles	Chromosome
1	<i>Xcfd21</i>	3	1D
2	<i>Xcfd20</i>	3	1D
3	<i>Xcfd27</i>	2	1D
4	<i>Xgdm35</i>	4	2D
5	<i>Xgwm296</i>	3	2D
6	<i>Xgwm261</i>	3	2D
7	<i>Xgwm515</i>	3	2D
8	<i>Xcfd11</i>	4	2D
9	<i>Xcfd44</i>	5	2D
10	<i>Xcfd53</i>	4	2D
11	<i>Xgdm8</i>	4	3D
12	<i>Xgwm314</i>	3	3D
13	<i>Xgwm645</i>	3	3D
14	<i>Xgwm383</i>	3	3D
15	<i>Xcfd4</i>	3	3D
16	<i>Xgdm72</i>	4	3D
17	<i>Xgdm125</i>	4	4D
18	<i>Xgdm129</i>	2	4D
19	<i>Xgwm194</i>	2	4D
20	<i>Xgwm192</i>	3	4D
21	<i>Xcfd19</i>	3	4D
22	<i>Xcfd10</i>	4	5D
23	<i>Xcfd358</i>	2	5D
24	<i>Xgdm193</i>	3	5D
25	<i>Xgdm68</i>	2	5D
26	<i>Xcfd49</i>	4	6D
27	<i>Xcfd5</i>	4	6D
28	<i>Xcfd37</i>	3	6D
29	<i>Xgdm141</i>	2	6D
30	<i>Xgdm132</i>	5	6D
31	<i>Xgwm469</i>	2	6D
32	<i>Xgwm111</i>	6	7D
33	<i>Xgwm428</i>	2	7D
34	<i>Xgwm44</i>	2	7D
35	<i>Xcfd31</i>	2	7D

average of 2.9 alleles per locus. The number of alleles showing significant association with each of the traits investigated at unadjusted P=0.01 and P=0.05 is given in Table 2. In all 46 marker-character combinations were

Table 2. Number of markers with significant association with each character

Character	P = 0.01	P = 0.05
Leaf rust resistance	18	32
Septoria tritici blotch resistance	6	17
Tan spot resistance	-	2
Green bug resistance	4	7
Hessian fly resistance	4	11
Wheat curl mite resistance	5	9
Nematode reaction	3	13
K ⁺ concentration	3	11
Na ⁺ concentration	3	9
Total	46	111

associated at 1 percent and 111 at 5 percent level of significance. The number of alleles associated at P=0.01 ranged from none for tan spot to 17 for leaf rust resistance with an average of 5.2 alleles per character as compared to expectation of 1.02 to be associated by random chance, at 1 per cent level of significance. About one third *i.e.* 18 of 46 at P = 0.01 and 32 of 111 at P = 0.05 involved resistance to leaf rust. The conservative test of significance, however, identified only one marker-character combination as significant. This only significant case also involved leaf rust resistance to be associated with marker *Xgdm35-180* located on chromosome 2D. Of the three quantitative characters one QTL was detected for nematode resistance and one allele at *Xcfd11* locus showed association with resistance to high salt (Na⁺ and K⁺) concentration with a LOD score of 2.5.

A significant magnitude of linkage disequilibrium was observed for pairs of microsatellite markers evenly distributed throughout the genome. Lubers *et al.* [23] also reported high levels of linkage disequilibrium between loci in a sample of germplasm collection of *Ae. tauschii*. The germplasm pool of many local collections from different geographic regions is thus expected to harbor such equilibrium. A relatively large number of marker pairs in disequilibrium involved chromosomes 2, 3 and 6 which could be the result of preferential segregation already reported to be operative in this species [24]. In light of this disequilibrium it would thus be expected to find at least fitness related loci to be associated with markers. Furthermore the markers included in this study categorized accessions on the patterns of their evolutionary history and expected divergence. All the accessions with resistance to leaf

rust were clustered in a group largely represented by stocks from Iran where resistance to this disease is expected to have evolved. The diverse collections from Iran were present in both of the two major groups whereas relatively related stocks from Afghanistan and Pakistan clustered in one group. It thus is apparent that these markers represent parts of chromosomes, which can be expected to show association with functionally important segments of the genome of this species.

Comparative mapping in donor accessions and derived germplasm lines

The markers associated with each character along with the probability of observed chi-square is given in Table 3. It is apparent that out of 46 cases significant through usual level of significance only one is significant after the adjustment of probability for multiple tests. The location of resistance genes on specific chromosomes and their association with the markers located on same chromosome detected through usual level of significance *i.e.*, P = 0.01 is given in Table 4. The sample of germplasm included in this investigation is known to carry at least three genes for resistance to leaf rust and four to Hessian fly but still only one case of marker-character association attained statistical significance through conservative test. Only one of the known major genes *Lr39* was declared to be associated with marker *Xgdm35* through conservative test. The donor accessions of resistance genes along with recipient parents and germplasm lines of winter wheat developed by transferring the specific resistance genes were also assayed to check the marker allele similarity in germplasm resources and the donor lines. Five accessions of *Ae. tauschii* (TA1644, TA1642, TA1695, TA2452 and TA2473) were resistant to Hessian fly whereas seven (TA1649, TA2460, TA2450, TA2541, TA2470, TA2397 and TA749) were resistant to leaf rust pathogen. However one accession TA1695 was also resistant to pest green bug. The presence of specific marker allele in germplasm lines and donor accessions in contrast to the recipient variety of wheat would suggest the association of resistance gene, at least with such coexisting marker allele. The DNA fragment amplified using marker *Xgdm35*, which shows the strongest association with leaf rust resistance, is present in donor accession, TA2460 and has been added to wheat variety to produce WGRC10. Likewise the fragments amplified with primer pair *Xgwm261-200* show exact correspondence between donor and respective germplasm lines but remain undetected through conservative test in spite of adequate polymorphism at marker and disease controlling loci.

Table 3. Markers with significant [P=0.01] association for each character

Character	Marker	P-value	Chromosome
Leaf rust resistance	<i>Xgdm35</i> (180)	0.00008	2D
	<i>Xgwm 383</i> (190)	0.0007	3D
	<i>Xcfd4</i> (270)	0.0025	3D
	<i>Xgwm 645</i> (300)	0.0010	3D
	<i>Xgwm 192</i> (150)	0.0032	4D
	<i>Xgwm 261</i> (200)	0.0027	2D
	<i>Xgwm 515</i> (120)	0.0037	2D
	<i>Xgwm 192</i> (170)	0.0028	4D
	<i>Xcfd19</i> (250)	0.0069	4D
	<i>Xcfd19</i> (300)	0.0076	4D
	<i>Xgwm 296</i> (120)	0.0085	2D
	<i>Xgwm 296</i> (150)	0.0091	2D
	<i>Xgwm 358</i> (170)	0.0099	5D
	<i>Xgwm 645</i> (150)	0.0115	3D
	<i>Xgdm132</i> (250)	0.0116	6D
	<i>Xgdm125</i> (120)	0.0133	4D
<i>Xcfd53</i> (180)	0.0134	2D	
<i>Xgwm 111</i> (200)	0.0179	7D	
Septoria tritici blotch resistance	<i>Xgwm296</i> (120)	0.0019	2D
	<i>Xgwm296</i> (150)	0.0020	2D
	<i>Xcfd53</i> (220)	0.0025	2D
	<i>Xgdm35</i> (300)	0.0043	2D
	<i>Xgwm314</i> (190)	0.0083	3D
Green bug resistance	<i>Xgwm296</i> (120)	0.0058	2D
	<i>Xcfd49</i> (300)	0.0057	6D
	<i>Xcfd10</i> (300)	0.0093	5D
	<i>Xgwm111</i> (130)	0.0089	7D
Hessian fly resistance	<i>Xcfd19</i> (250)	0.0070	4D
	<i>Xgwm261</i> (200)	0.0023	2D
	<i>Xcfd4</i> (250)	0.0043	3D
	<i>Xgwm192</i> (180)	0.0133	4D
White curl mite resistance	<i>Xgwm314</i> (150)	0.0009	3D
	<i>Xcfd49</i> (180)	0.0041	6D
	<i>Xgwm314</i> (190)	0.0044	3D
	<i>Xgwm383</i> (200)	0.0045	3D
	<i>Xcfd 31</i> (200)	0.0144	7D
Nematode resistance	<i>Xgwm192</i> (150)	0.0030	4D
	<i>Xgwm645</i> (300)	0.0053	3D
	<i>Xgwm192</i> (170)	0.0078	4D
K ⁺ concentration	<i>Xcfd11</i> (220)	0.0009	2D
	<i>Xgdm125</i> (120)	0.0139	4D
	<i>Xgdm125</i> (150)	0.0142	4D
Na ⁺ concentration	<i>Xcfd11</i> (170)	0.0010	2D
	<i>Xgdm193</i> (130)	0.0029	5D
	<i>Xgwm296</i> (120)	0.0143	2D

Amplified fragment size is given in parentheses

Ae tauschii accessions used in this study are known to be donor of at least three *Lr* genes, out of which only one *Lr39* to be a candidate and already reported tightly linked with marker *Xgdm35* [25]. The accessions in present investigation were categorized as resistant and susceptible non-specifically i.e. without any identity of corresponding resistance gene for diseases and insects. Each resistant group for leaf rust or Hessian fly, as an instance, represents the effect of several independent genes e.g. three for leaf rust and four for Hessian fly which apparently dilutes the association of a marker with the effect of gene actually linked with the marker. It is due to this genetic heterogeneity that even the pairs of markers and genes known to be located on the same chromosomes could reach only low level of significance that would normally remain ignored at conservative level of significance. It is a strong indicator of the influence that genetic heterogeneity can have on the detection and precise location of genes controlling quantitative characters which are supposed to be influenced by several loci. Locus and allelic heterogeneity which are common in complex diseases can produce drastic reduction in power to detect linkage disequilibrium [26, 27].

Distribution of marker alleles in germplasm pool

Out of all possible 5151 pairs of marker alleles, 76 pairs showed correlated distribution even with the conservative test of significance with adjusted probability. Forty of 76 significantly associated pairs of alleles at conservative test at one-percent level of significance involved a block of more than two markers. Two alleles at locus *Xgwm296* and one at *Xgwm383* showed GPD with a combination of four alleles. A greater frequency of pairs in disequilibrium involved markers located on chromosome 2, 3, and 6 of D-genome. The cluster analysis revealed two major clusters and all the collections from Afghanistan and Pakistan falling in one group. The accessions from Iran were distributed in both the groups confirming wide diversity among Iranian collections. Twenty of the 25 accessions with leaf rust resistance genes were clustered in group predominated by collections from Iran where genes for leaf rust resistance are expected to have evolved. Both the clusters, however, had comparable frequency of resistance genes with respect to resistance to *Septoria tritici* blotch, green bug and Hessian fly.

The present investigation provides indirect evidence suggesting the existence of many biologically pertinent marker-gene associations that, in fact,

Table 4. Markers with significant [$P = 0.01$] association with genes mapped on the same chromosomes in the *Ae. tauschii* genotypes studied

Character	Marker	Gene	Chromosome
Leaf rust	<i>Xcfd19-250</i>	<i>Lr21</i>	1D
	<i>Xcfd19-300</i>	<i>Lr42</i>	1D
	<i>Xgdm35-180</i>	<i>Lr39, Lr22a</i>	2D
	<i>Xgwm261-200</i>	<i>Lr39, Lr22a</i>	2D
	<i>Xgwm515-120</i>	<i>Lr39, Lr22a</i>	2D
	<i>Xcfd4-270</i>	<i>Lr32</i>	3D
	<i>Xgwm645-150</i>	<i>Lr32</i>	3D
Hessian fly	<i>Xcfd19-250</i>	H 22	1D
	<i>Xcfd4-250</i>	H 24	3D
	<i>Xgwm192-180</i>	H 26	4D

remained statistically undetected. Significantly associated marker *Xgdm35* to *Lr39*, other markers viz. *Xgwm261* represented a fragment (allele) each of which was amplified in donor accessions and the corresponding germplasm line, though these were absent in recipient susceptible varieties. Based on our study, comparative distribution of associations across D genome shows chromosome 2D is associated with resistance to several stress factors. Fine genetic mapping of defense-related loci in this species observed clusters of defense-related genes on 2D chromosome [28]. About 1/3 of total loci mapped on 2D chromosome were defense related, the most among all chromosomes. The association analysis thus not only provided a probable location of resistance genes but also suggested the conserved distribution of defense related genes. A detailed analysis of chromosome 2D may reveal the mechanism of evolution and operation of resistance genes in wheat.

Most of the marker-gene associations could, in fact, be detected only through usual error rate of five percent. But even at this level of significance, a particular chromosome 2D was identified to be associated with multiple resistance which also is supported by fine genetic mapping of defense-related genes in this species. Existence of clusters of QTL on different chromosomes for many quantitative characters have been reported [8]. Ford-Lloyd *et al.* [29] also observed the existence of co-adaptive gene complexes in rice which showed association with blocks of markers being maintained on different chromosomes. The analysis has

thus potential use in identification of such regions of genome that might play key role in the expression of quantitative traits. The concept of association mapping in plant populations may not, as yet be useful for marker assisted selection but can serve the purpose of initial screening and categorization of germplasm for at least extracting appropriate germplasm accessions for crop improvement.

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