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T. Krugman · O. Levy · J. W. Snape · B. Rubin
A. Korol · E. Nevo

Comparative RFLP mapping of the chlorotoluron resistance gene (*Su1*) in cultivated wheat (*Triticum aestivum*) and wild wheat (*Triticum dicoccoides*)

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Abstract Chlorotoluron is a selective phenylurea herbicide widely used for broad-leaved and annual grass weed control in cereals. Variation in the response to chlorotoluron (CT) was found in both hexaploid bread wheat (*Triticum aestivum* L.) and wild tetraploid wheat (*Triticum dicoccoides* KöRN.). Here, we describe the comparative mapping of the CT resistance gene (*Su1*) on chromosome 6B in bread and wild wheat using RFLP markers. In bread wheat, mapping was based on 58 F_4 single-seed descent (SSD) plants of the cross between a genotype sensitive to chlorotoluron, 'Chinese Spring' (CS), and a resistant derivative, the single chromosome substitution line, CS ('Cappele-Desprez' 6B) [CS (CAP6B). In *T. dicoccoides*, mapping was based on 37 F_2 plants obtained from the cross between the CT-susceptible accession B-7 and the resistant accession B-35. Nine RFLP probes spanning the centromere were chosen for mapping. In bread wheat *Su1* was found to be linked to *α-Amy-1* (9.84 cM) and *Xpsr371* (5.2 cM), both on the long arm of 6B, and *Nor2* (2.74 cM) on the short arm. In wild wheat the most probable linkage map was *Nor2-Xpsr312-Su1-Pgk2*, and the genetic distances between the genes were 24.8cM, 5.3cM, and 6.8cM, respectively. These results along with other published map data indicate that the linear order of the genes is similar to that found in *T. aestivum*. The results of this study also show that the *Su1* gene for differential response to chlorotoluron has evolved prior to the domestication of cultivated wheat and not in response to the development and use of chemicals.

Key words Herbicide resistance · Chlortoluron · RFLP · *Triticum dicoccoides* · *Triticum aestivum*

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T. Krugman (✉) · A. Korol · E. Nevo
Institute of Evolution, Haifa University, Mt. Carmel, Haifa 31905, Israel

J.W. Snape
John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

O. Levy · B. Rubin
Faculty of Agricultural food and environmental sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel

Introduction

Chlorotoluron (CT) is a selective phenylurea herbicide widely used for broad-leaved and annual grass weed control in cereals. These herbicides mediate their effect on weeds by interfering with electron transport in photosystem II (PSII). Variation in the response to chlorotoluron was found in hexaploid bread wheat (*Triticum aestivum* L.) and in wild tetraploid wheat (*Triticum dicoccoides* KöRN.), the latter being the progenitor of cultivated wheat, from different ecogeographical areas of Israel (Snape et al. 1991b, c).

The genetic control of tolerance to chlorotoluron in bread wheat is determined by a single major gene, *Su1*, located on the short arm of chromosome 6B (Snape et al. 1991a), but neither the mechanism of resistance nor any gene product has been identified. Moreover, the physiological mechanism of the varietal selectivity of wheat to chlorotoluron is still not clear (Cabanne and Snape 1993). Studies of chlorotoluron metabolism in tolerant varieties indicate that detoxification by ringmethyl oxidation represents the probable mechanism, while in susceptible varieties, metabolism is generally slow and restricted to *N*-mono-dealkylation (Ryan and Owen 1983). Current opinions are that the detoxification of the herbicide in bread wheat is correlated with the activity of cytochrome P_{450} mixed-function oxidases (Gonneau et al. 1988; Jones and Caseley 1989; Jones 1991), this has also been indicated in wild wheat (Levy 1995). The chlorotoluron-tolerant phenotype in wild emmer wheat has been correlated with ecological factors and allozyme markers located on chromosome 6B (for example, *Adh*, *Est-4* and *Got*) (Nevo et al. 1992). These findings imply

that chlorotoluron resistance has a similar mode of action in bread and wild wheat. However, the resistant alleles present in wild wheat might be different and could, for example, provide cross resistance to several more herbicides (Snape et al. 1991a). Revealing the precise genetic control of herbicide response in cultivated and wild wheat is, therefore, essential for understanding the mechanism and evolution of chlorotoluron resistance in wheat.

A detailed genetic map, based largely upon restriction fragment length polymorphism (RFLP), has now been constructed for bread wheat and comprises over 800 loci dispersed over the whole genome (Devos and Gale 1993). Fine mapping of a target gene is the prerequisite for its physical mapping and subsequently for map-based cloning and for marker-assisted breeding (Tanksley et al. 1989; Paterson and Wing 1993; Martin et al. 1993; Korol et al. 1994). When differential responses to herbicides occur, there is generally a necessity for new varieties to incorporate resistance. Since the gene product is unknown, elucidation of the location of the genes will greatly simplify the process. In this study we describe the comparative mapping of the *Su1* gene on chromosome 6B in bread and wild wheat using RFLPs as a preliminary to developing a marker-assisted selection method for breeding for resistance to chlorotoluron.

Materials and methods

Mapping populations

In bread wheat, mapping was based on 58 F_4 plants of the cross between a genotype sensitive to chlorotoluron, 'Chinese Spring' (CS), and a resistant derivative, the single chromosome substitution line, CS('Cappelle-Desprez 6B') [CS(CAP6B)] (developed by C. N. Law and A. J. Worland). The F_4 plants were derived by single-seed descent (SSD) so that each F_4 line represents a different F_2 plant. These single-chromosome recombinant lines were selected from F_3 families that had been classified on the basis of their response to CT treatment (Snape et al. 1991a). The parental lines and ditelosomics of CS (DT6BL, DT6BS) (Sears 1954) were used to ascertain chromosomal arm locations of the fragments identified by hybridization with the DNA probes as previously described by Sharp et al. (1989).

In *T. dicoccoides*, mapping was based on 37 F_2 plants obtained from the cross between the CT-susceptible accession B-7, and the resistant accession B-35. Both originated from individual plants of a wild population collected at the Yehudiyya site, Israel (described in Nevo and Beiles 1989).

Chlorotoluron treatment and resistance classification

The response to chlorotoluron was scored by estimating chlorophyll fluorescence induction kinetics decay. This was recorded following the exposure of detached leaves treated with chlorotoluron to a single turnover flash light (Benyamini et al. 1991). Measurements were carried out with a chlorophylol fluorometer (PAM, Walz, Effeltrich, Germany). Detached leaves, 15 cm long, were put in 15 ml test tubes containing 2 ml of reaction mixture [10^{-4} *M* chlorotoluron dissolved in 1% ethanol and 0.1% surfectant solution (Citowett, BASF Germany)]. Treated and control leaves were transferred to a growth chamber (25°C, 300 μE $m^{-2} s^{-1}$) for 2 h in order to allow complete distribution of the herbicide in the leaf, then recut under water and transferred to 2 ml of distilled water in the dark. Following a period of 15 min of dark adaptation, chlorophyll fluorescence measurements were taken immediately, 24 h and 48 h after treatment. The state when all reaction centres of PSII(RCII) are ready ("open"), F_0' was recorded using weak modulated light (measuring light). The peak of fluorescence induction (F_P) occurs after a short flash (8 ms) of strong actinic light allowing a single charge separation in most RCII while reducing the primary acceptor, Q_A (RCII are "closed"). During the dark period which follows the flash, Q_A is oxidized while delivering the electron to Q_B, the concentration of "closed" RCII is lowered, and as a consequence, the fluorescence signal declines (F_t), as described in Fig. 1. When PSII inhibitors are present (e.g. chlorotoluron), electron delivery to Q_B is prevented, thus resulting in inhibition of the fluorescence decay. F_t was determined for a period of 40 ms after the flash. Electron transport (ET) capacity is a parameter which quantifies the capacity of electron transport from Q_A to Q_B and quantifies by Fv. Fv is calculated as follows:

$$Fv = (Fp - Ft)/(Fp - Fo)$$

When PSII inhibitor is present, ET from Q_A to Q_B is prevented, resulting in inhibition of the signal decay (Fv ≈ 0.1), as compared to untreated control (Fv ≈ 1.0). After a recovery period in the dark, plants able to degrade the herbicide will restore all or part of their ET capacity and, consequently, the fluorescence signal recovers (Fv = up to 1.0):

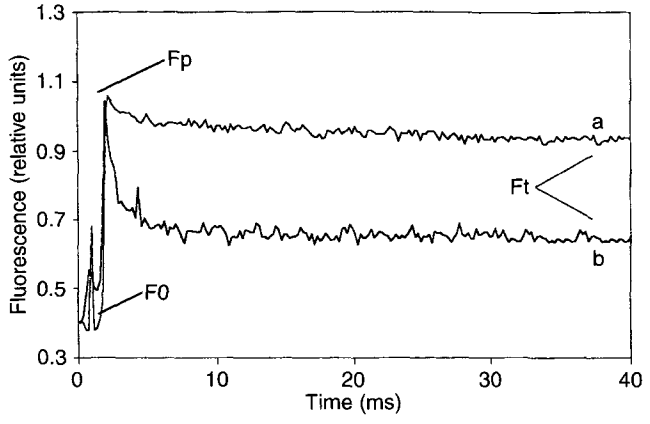
$$\text{Fluorescence Recovery (REC)} = (\text{Fv treated}/\text{Fv untreated})*100$$

Based on changes in fluorescence signal, recovery is calculated for each individual plant and used for the quantitative determination of herbicide resistance. Plants were regarded as resistant (*R*) when REC was over 50%. All plants with intermediate REC were regarded as resistant.

DNA isolation and Southern hybridization

Total DNA was extracted from leaves of plants using the method of Appels and Dvorak (1982). The DNA (8 μg) was digested with five separate restriction enzymes, *Hin*dIII, *Bam*HI, *Eco*RI, *Dra*I and *Taq*I following manufacturer's recommendations (New England, BioLabs, USA). Restriction fragments were separated by electrophoresis on 1% agarose gels, denatured and blotted onto nylon filters (Hybond-N, Amersham, UK). RFLP probes were radiolabeled by the random primer method of Feinberg and Vogelstein (1984). Prehybridization

Fig. 1 Fluorescence induction curve obtained from wheat leaves after 15 min of dark adaptation. *a* Leaf trearted with 10^{-4} chlortoluron for 2 h, *b* untreated control. *Fo*; all reaction centers of PSII(RCII) are ready; recorded using weak modulated light. *Fp*; the peak of fluorescence induction after a short flash (8 μs) of actinic light. *Ft*; during the dark period, Q_A is oxidized while delivering the electron to Q_B, and the fluorescence signal declines; determined 40 ms after the flash



and hybridization of [^{32}P]-labelled probes with the DNA on filters were performed in 50% formamide solution at 42°C. After hybridization with *pTα71*-IGS, the filters were washed at a stringency of 0.1 × SSPE at 65°C and were exposed to X-Ray film (Kodak-XOmat film) for 30 min–1 h. After hybridization with all other probes, the filters were washed with 0.5 × SSPE, 0.1 × SDS at 65°C and exposed to film for 2 days.

RFLP probes

Nine RFLP probes spanning the centromere were chosen according to their location on the chromosome 6B linkage map (Devos and Gale 1993) and based on the provisional location of *Su1* near to the centromere (Snape et al. 1991a): three known function probes, *α-Amy-1-3*, sub-fragment cDNA clone 2128 (described in Lazarus et al. 1985, kindly provided by D.C. Baulcombe); *Nor2*, wheat rDNA intergenic spacer (IGS)–3.0-kb *Taq*I digested from the recombinant plasmid *pTA71* (described in Gerlach and Bedbrook 1979, kindly provided by M. O'Dell); *Pgk2* (*Xpsr 141*), wheat phosphoglycerate kinase (Longstaff et al. 1989); three anonymous cDNA probes–wheat leaf cDNAs *Xpsr106*, *Xpsr149*, *Xpsr88*; and three gDNA probes, *Xpsr312*, *Xpsr371*, and *Xpsr627* (kindly provided by M. D. Gale).

Linkage estimation

Triticum dicoccoides KöRN

Linkage analysis was performed using MAPMAKER 3.0 b (Lander et al. 1987). Two-point analysis was performed first to determine the maximum-likelihood recombination fraction and the associated LOD scores of the six pairs of 3 loci and the chlorotoluron resistance locus. To detect linkage we used a threshold LOD score of 3.0. The multi-point command "ripples" was used to establish the framework order of markers. The Kosambi mapping function was used to calculate map distances in centiMorgans (cM).

Triticum aestivum

Frequencies between loci were calculated as described by Snape (1988) with minor modifications, where a maximum likelihood estimation of r was obtained by numerical iteration. As described above, the progeny obtained after three generations of SSD was estimated for CT resistance, and equal numbers of resistant (R) and sensitive (S) genotypes were scored for their alleles at the molecular markers. Hence, the expected ratio of R:S as well as their real proportion was not 1:1. Thus, the expected frequencies of marker classes were normalized within the two resistance groups by their totals in order to obtain the expectations corresponding to the experimental designs used.

Results

Chlorotoluron response

Chlorophyll fluorescence is widely used as a probe of the processes of photosynthesis *in vivo*, providing information on inhibition or damage to electron transfer from photosystem II (PSII) of individual plants (Bolhar-Nordenkampf et al. 1989; Benyamini et al. 1991). Photosynthesis was inhibited by 80% in all of the treated plants immediately after chlorotoluron treatment (REC ≅ 20%) as compared to untreated plants. Resistant plants showed a 68–97% recovery 24 h and/or 48 h after treatment, while susceptible plants were still inhibited 48 h after treatment, with a recovery of only 15–40%. For segregation analysis, all plants with REC values over 50% were regarded as being resistant.

The segregation ratio for chlorotoluron resistance in the *T. dicoccoides* F_2 progeny was 14S:24R ($\chi^2 = 2.42$, $P > 0.5$, for 1:3 segregation), indicating that the difference between the resistance and susceptible responses is, as expected, due to a single major locus.

RFLP and linkage analysis

The parents of each cross were screened for polymorphism using the different restriction enzyme/probe combinations. Polymorphism between the *T. aestivum* parents was detected for *α-Amy-1/Hin*dIII, *Nor2 /Taq*I and *Xpsr371/Eco*RI. Polymorphism between the *T. dicoccoides* parents was detected by *Nor2/Taq*I, *Pgk2* (*Xpsr141)/Hin*dIII and *Xpsr312/Eco*RI. Chromosome and chromosomal arm locations of polymorphic bands in *T. aestivum* were determined using ditelosomic lines of CS (Figs. 2,4), whereas in *T. dicoccoides* the positions of the loci were determined by progeny tests, where the polymorphic probes were hybridized to DNA of the segregating populations (Table 2). As expected, in both wild and bread wheat, each plant displayed either one (in homozygotes) or both band patterns (in heterozygotes) of the parental 6B polymorphic fragments (Tables 1, 2).

The rDNA probe detected polymorphism in both crosses. In bread wheat the nucleolus organizer regions (*Nor*) containing the 18S.26S ribosomal DNA clusters have been detected by in situ hybridization on chromosome arms 1AS, 1BS (*Nor1*), 5DS (*Nor3*), 6BS (*Nor2*) and 7DL (Mukai et al. 1991) and on 1BS and 6BS in wild wheat (Appels and Dvorak 1982; Flavell et al. 1986). The fragment profile of CS consisted of a 3.1-kb fragment for *Nor1* and 2.6-kb and 2.8-kb fragments for *Nor2*. Here, CS(CAP6B) differed only in the 2.2-kb fragment from CS (Fig. 2). In *T. dicoccoides* the fragment profile of each

Fig. 2 Southern blot hybridization of *T. aestivum Taq*I digest DNA from the cross between CS (S) (*lane 1*) and CS (CAP6B) (R) (*lane 2*) and their F_4 SSD lines, probed with rDNA gene *pTA71*- IGS. *Lanes 3, 4, 6, 7* CT susceptible with CS band pattern, *lanes 8–10* CT resistant with CAP band pattern, *lane 5* CT-resistant heterozygote (note that the 2.2-kb band of *Lane 2, 5, 8* is weak)

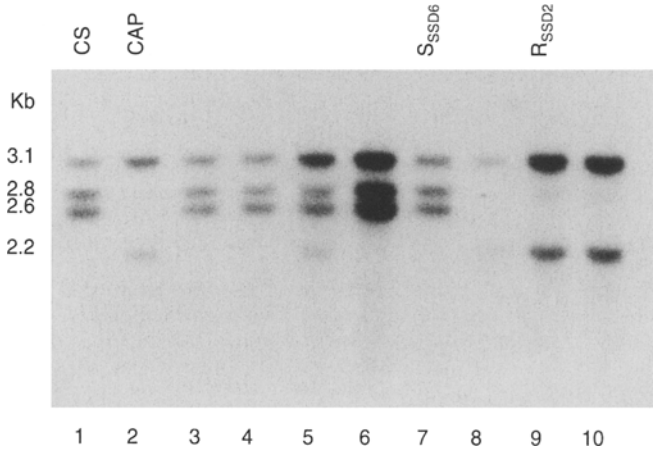


Table 1 Segregation of RFLP markers and chlorotoluron response in *T. aestivum* F_4 SSD lines (*RE* restriction enzyme, *R* resistant, *S* sensitive

Locus	Polymorphic fragments (kb)	CT response R	S	n	Recombination %
Nor2	CAP 2.2 kb	24[a]	1	58	2.74 ± 1.65
RE: *Taq*I	CAP/CS	5	1		
	CS 2.6;2.8 kb	1	26[a]		
α-Amy-1	CAP3.0;6.5 kb	20[a]	4	57	9.84 ± 3.36
RE:*Hin*dIII	CAP/CS	7	3		
	CS 3.5;4.0 kb	3	20[a]		
Xpsr371	CAP 7.0 kb	20[a]	0	50	5.15 ± 2.52
RE:*Eco*RI	CAP/CS	3	4		
	CS 6.5 kb	1	22[a]		

[a] Parental type

Table 2 Segregation of RFLP markers and chlortoluron response in *Tdicoccoides* F_2 progeny

Locus	Polymorphic fragments (kb)	CT response R	S	*n*
Nor2	B35 2.8 kb	4[b]	2	
RE: *Taq*I	B35/b7	19	3	37
	B7 3.1 kb	1	8[b]	
Xpsr312	B35 6.0 kb	21	1	
RE: *Eco*RI	B7 Null	0	11	33
Pgk2 (*Xpsr141*)	B35 Null	10	0	
RE: *Hin*dIII	B7 3.5 kb[a]	1	21	32

[a] Homozygotes and heterozygotes (dominant allele)
[b] Parental type

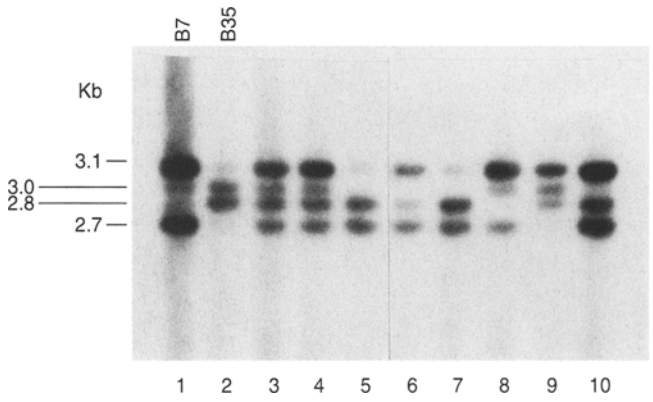


Fig. 3 Southern blot hybridization of *T. dicoccoides Taq*I digest DNA from the cross parents B-7 (S) and B-35 (R) (*lanes 1, 2*) and their F_2 progeny (lanes 3–10), probed with rDNA gene *pTA71-IGS*. *Lanes 1, 2* B-7 (S), B-35 (R) *Lanes 3–10* band patterns of *Nor1* and *Nor2* in F_2 progeny: *lanes 3, 4* double heterozygote, *lanes 5, 7* double homozygotes, *lanes 6, 8, 9, 10* heterozygote in one gene and homozygote in the other gene

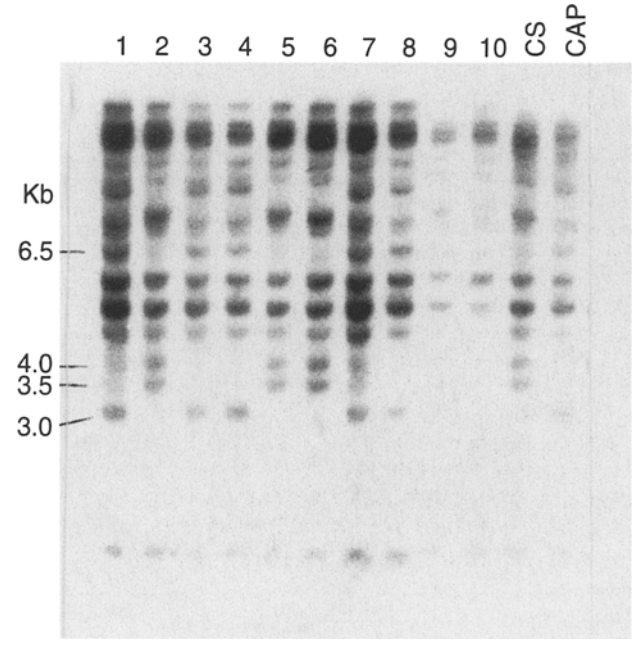


Fig. 4 Southern blot hybridization of *T. aestivum Hin*dIII digest DNA from the cross between CS (S) and CS (CAP6B) (R) and F_4 SSD lines (*lanes 1–8*), probed with α-Amy-1 (2128). Ditelosomics DT6S (*lane 9*) and DT6L (*lane 10*) were used to determine the location of the 6B polymorphic bands

of the parents consisted of two bands each originating from *Nor1* or *Nor2*. The B-37 profile consisted of 2.7-kb and 3.1-kb bands, and B-35 of 3.0-kb and 2.8-kb bands. Allelism at each locus was deduced from double homozygotes observed in the F_2 (3.0 kb/3.1 kb and 2.7 kb/2.8 kb), in which each of the two fragments originated from a different *Nor* locus (Fig. 3). Hence, the 3.1-kb/2.8-kb bands are alleles of one locus, and the 3.0-kb/2.7-kb bands are alleles of the other locus. Segregation of the 2.8-kb/3.1-kb fragments with chlorotoluron response, among the 37 F_2 progeny tested, gave the distance between the genes as 24.8 cM (by multipoint analysis). Hence, this locus is the *Nor2* locus on 6B, while the 3.0-kb/2.7-kb locus segregated independently of the resistance gene (see Fig. 3, Table 2), and hence is on 1B.

Southern hybridization of the *α-Amy-1* cDNA clone 2128 revealed a multiband pattern originating from each of the three homeoloci of group 6 chromosomes (Cheung et al. 1991). The CS 6BL fragments were 3.5 kb and 4.0 kb in size, whereas the CS (CAP 6B) bands were 6.5 kb and 3.0 kb (Fig. 4). The *Xpsr371/Eco*RI combination revealed six fragments, but the two parents differed only in a 6.5-kb, CS, and a 7.0-kb CS (CAP6B) fragment.

In wild wheat, loci *Pgk2 (Xpsr141)* and *Xpsr312* showed a dominant mode of inhertitance, i.e. polymorphic fragments were present in only one parent (Table 2).

Taken together, these results show that the chlorotoluron resistance gene in *T. dicoccoides* is located on chromosome 6B, which may indicate that it is the same locus as *Su1* in *T. aestivum*. Hence, it will be referred to as *Su1'*. Using two-point analysis, we found '*Su1*' and *Xpsr312* to be closely linked (5.6 cM, LOD = 6.13), while the other markers and chlorotoluron resistance

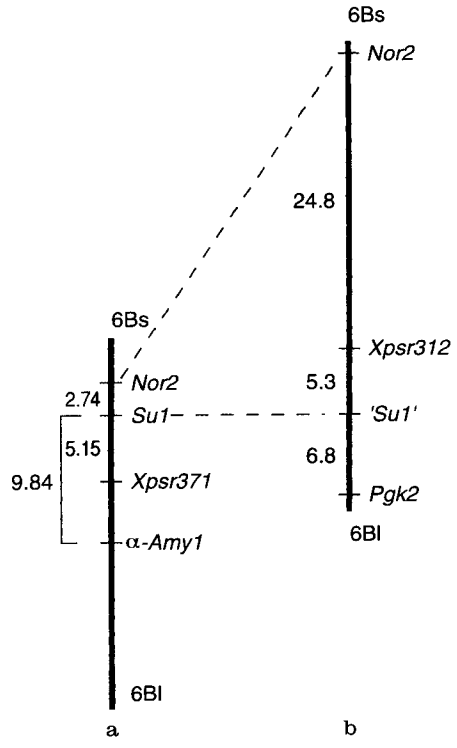


Fig. 5a,b Comparative linkage maps of 6B chromosome. **a** *T. aestivum* based on F_4 of the cross CS × CS (CAP6B), **b** *T. dicoccoides* based on F_2 of the cross B-7 × B-35. The genes and markers are given on the *right side* of the chromosome; map distances are given in Centi Morgans (CM) on the *left side*

gene were unlinked (LOD < 2.5). The multipoint command "ripples" was used to establish the framework order of markers, showing that the order of loci was *Nor2-Xpsr312-'Su1'-Pgk2*, with genetic distances between the genes of 24.8 cM, 5.3 cM, and 6.8 cM, respectively (Fig. 5b).

Discussion

Herbicide resistances are considered to be good candidates for plant biotechnological modification, since they are a potentially valuable agronomic trait, and are generally determined by single major genes (Oxtoby and Huges 1989). In this study, a method for herbicide resistance identification and quantification in detached leaves of individual plant was used. The fluorescence method can detect variation in response to PSII inhibitor herbicides, as demonstrated in this study. It is rapid, non-destructive, reproducible and can be conducted with whole plant and excised leaves under both laboratory and field conditions. A similar method was used with the same herbicide in blackgrass (*Alopecurus myosuroides* L.) (Van Oorschot and Van Leeuwen 1992), but unlike the usual fluorescence methods our method requires only 40 ms in order to quantify the plant response to chlorotoluron. Since the method is non-destructive, the same individual plant was used for resistance quantification and for the RFLP analysis. Chlorotoluron-treated leaves from resistant and susceptible plants have the same fluorescence signal immediately after treatment due to binding of the herbicide to the D_1 protein on the thylakoid membrane. However, after a recovery period the signal from the resistant plants returned to the control level. This phenomenon could be explained by the removal of the inhibitor from its binding site, probably by detoxification. CT detoxification in wheat by the activity of cytochrome P_{450} mixed-function oxidases has been said to be involved in the detoxification of chlorotoluron in wheat (Gonneau et al. 1988; Jones and Caseley 1989; Jones 1991). However, Cabanne and Snape (1993) found a similar metabolism for chlorotoluron in cell suspension cultures prepared from some of the SSD lines used in the present study. This contradiction may be due to the use of unsprayed F_4 SSD plants (which were still segregating in their response to CT) in the preparation of the cell cultures.

RFLP markers were identified linked to the *Su1* gene in *T. aestivum: Nor2* on the short arm and *Xpsr371* and *α-Amy-1* on the long arm. In *T. dicoccoides Xpsr312* was linked to *'Su1'*, and the order of the other markers along the 6B chromosome was determined to be *Nor2-Xpsr312-'Su1'-Pgk2* (see Fig. 5). In a map of 6B of hexaploid wheat *Xpsr312* is 2.6 cM from *Nor2* (Hart et al. 1993), and data from other crosses of *T. aestivum* (for example doubled haploid lines from the cross of 'Highbury' × 'Sicco') indicate that *Pgk2* is 10 cM from *a-Amy-1* (J.W. Snape, unpublished results). This indicates that the linear order of the genes along the 6B chromosome is similar to that found in *T. dicoccoides: Nor2-Xpsr312-'Su1'-Pgk2*. However, although the gene order is similar, the genetic distance of *'Su1'-Nor2* in wild wheat is tenfold higher than in bread wheat. This difference in map distance between the two species may be a function not only of the chromosomal location of the markers but also of the genotypes studied, environmental conditions (Israel and England) and the size of the segregating populations (which was rather small in the wild wheat population of our study). Consequently, the distances represent approximations and not absolutes, as found also by Chen et al. (1994). Noteworthy, significant differences in recombination values between crosses were found earlier in wheat (Pogna et al. 1993).

In both bread and wild wheat a consistent non-correspondence is observed between the physical and genetic locations of genes along the chromosomes. This discrepancy between the genetic map and physical map can vary between the species we used in our crosses. Moreover, it is the regions with low ratio of genetic/physical distances which manifest the highest genetic variation and strongest reaction to recombination-inducing environmental conditions (Korol et al. 1994). Comparative analysis of recombination in cultivated wheat and its wild progenitor deserves more attention

since it provides a system for examining the evolution of large genomes under domestication, especially in association with polyploidization.

Our results together with the indications that the detoxification of the herbicide is correlated with the activity of cytochrome P_{450} mixed-function oxidases in both bread wheat (Gonneau et al. 1988; Jones and Caseley 1989; Jones 1991) and wild wheat (Levy 1995) imply that a similar genetic control of chlorotoluron resistance exists in bread and wild wheat and that both species show response polymorphisms. Intriguingly, these differences must reflect genetical variation which already exists within the species and that the *Su1* gene for differential response to chlorotoluron has evolved prior to the domestication of cultivated wheat and not in response to the development and use of chemicals.

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