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MOLECULAR TAGGING AND CONFIRMATION OF *THINOPYRUM DISTICHUM* CHROMOSOMES THAT CONTRIBUTE TO ITS SALT TOLERANCE

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ABSTRACT

An earlier study has shown that *Thinopyrum distichum* chromosomes $3J_1^d$, $4J_1^d$ and $5J_1^d$ are primarily responsible for its salt tolerance while $2J_1^d$ and a group 7J chromosome may also contribute. Presently, RFLP polymorphisms and C-bands were used to develop disomic addition lines in triticale for each target chromosome barring $7J_1^d$. An initial attempt to find PCR-based markers for the critical chromosomes employed sequences of mapped wheat cDNA loci to derive primers for the amplification of *Thinopyrum* homoeoloci. This approach met with limited success and when the various addition stocks became available these were used to detect *Thinopyrum*-specific RAPDs that could be converted into SCAR markers. Using the SCAR markers, a range of *Th. distichum* – triticale secondary hybrids with different combinations of the critical chromosomes were selected and employed in a salt tolerance test. The results confirmed those of the earlier study and showed that certain chromosome combinations rather than single chromosomes were required for elevated levels of salt tolerance. Chromosomes $2J_1^d$ plus $3J_1^d$ appeared to be the only combination of two chromosomes at a time that produced a notable effect. Addition of either of the combinations $2J_1^d$, $3J_1^d$ and $5J_1^d$ or $3J_1^d$, $4J_1^d$ and $5J_1^d$ to triticale produced salt tolerance approaching that of the primary *Th. distichum*/ triticale amphiploid. Attempts to further characterize critical chromosome regions and to develop plant material with introgressed tolerance should therefore focus on the J_1^d genome chromosomes 2, 3, 4 and 5.

Keywords: Abiotic stress tolerance - alien addition lines - intergeneric hybridization - gene transfer.

INTRODUCTION

Species of the section Thinopyrum of the genus *Thinopyrum* are maritime grasses of which *Thinopyrum distichum* (Thunb.) Löve (2n = 28 = $J_1^dJ_1^dJ_2^dJ_2^d$) is indigenous to South Africa (Pienaar, 1990). The ability of members of the genus to thrive in harsh, highly saline environments prompted numerous past attempts to understand the basis of its adaptability and to transfer hardiness genes to the cultivated grasses (Pienaar, 1990; Colmer *et al.,* 2006). Salt tolerance is a complex trait, not only because of the different mechanisms involved but also because of variation in the stress condition itself (Volkmar *et al.,* 1998; Singh and Chatrath, 2001). In the Triticeae the polygenic basis of salt tolerance was demonstrated by numerous studies involving both cultivated and wild grasses (Littlejohn, 1988; Omielan *et al.*, 1991; Gregorio and Senadhira, 1993; Ashraf, 1994; Colmer *et al.,* 2006) and it may involve similar genetic mechanisms in the different species (Zhong and Dvořák, 1995a; Colmer, 2006). Forster *et al.* (1988) concluded that chromosome $5J_1$ of *Th. bessarabicum* (Savul & Rayss) Löve has a major effect on salt tolerance. In *Th. elongatum* (Host) DR Dewey tolerance of sudden salt stress was principally controlled by chromosomes $3J_1^e$ and $5J_1^e$ whereas tolerance of gradually imposed salt stress was principally controlled by chromosomes $3J_1^e$, $4J_1^e$ and $5J_1^e$ (Zhong and Dvořák, 1995b). The higher level of salt tolerance of the wild parent was expressed in hybrids of *Th. elongatum* with common wheat (*Triticum aestivum* L.) (Omielan *et al.*, 1991) and *Th. bessarabicum* with durum wheat (*T. turgidum* L.) (King *et al.,* 1997), whereas levels of salt tolerance comparable only to those in the *Triticum* parents were reported for hybrids of *Th. bessarabicum* (Gorham, 1988) and *Th. distichum* (Littlejohn, 1988) with common and durum wheat. However, primary hybrids of *Th. distichum* with tetraploid rye (*Secale cereale* L.) (Marais *et al.,* 1998) and hexaploid triticale (X *Triticosecale* Wittmack) (Marais and Marais, 1998) expressed high levels of salt tolerance. Marais *et al.* (1998) produced a partially diploid primary *Th. distichum*/tetraploid rye hybrid ($J_1^dJ_2^d$RR) that facilitated construction of a *Th. distichum* C-band karyotype (Marais and Marais, 2003). A study of testcross F_1 chromosome segregation patterns coupled with RFLP analysis allowed for the grouping of the *Thinopyrum* chromosomes into seven homoeologous pairs. Concomitant salt tolerance tests provided strong evidence that chromosomes $3J_1^d$, $4J_1^d$ and $5J_1^d$ primarily determined salt tolerance. It furthermore appeared likely that chromosome $2J_1^d$ also had an effect, albeit small. While the least conclusive, the data also suggested possible involvement of one of the two group 7 chromosomes.

Marais and Marais (1998) successfully hybridized *Th. distichum* with hexaploid triticale and backcrossed the C_1 hybrid (2n=70) to triticale. In the present study the secondary hybrids thus produced were used in the first stage of an attempt at marker-assisted introgression to triticale of chromosome regions that could improve its salt tolerance. For this purpose single chromosome addition lines were developed in triticale for those *Th. distichum* chromosomes that were previously implicated in salt tolerance. DNA polymorphisms uniquely associated with the *Thinopyrum* target chromosomes were then identified and converted into easier-to-use PCR-based markers. Preliminary data suggested that additions of the individual critical chromosomes to triticale had only small effects on the level of salt tolerance. These effects were difficult to quantify and were unlikely to be commercially useful.

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Also, the genes involved in a tolerance mechanism would not necessarily occur on the same chromosome. Consequently, it was decided to also test combinations of critical *Thinopyrum* chromosomes and attempt to confirm their effects and identify the smallest subset of target chromosomes that would have a marked effect on salt tolerance of triticale.

MATERIALS AND METHODS

Development of addition lines

Addition plants were derived in two ways: (i) Backcross F_1 97M1: *Th. distichum*/2* hexaploid triticale plants with 2n = 54-56 chromosomes (*Thinopyrum* cytoplasm) were backcrossed reciprocally to the triticale cultivar 'Rex' to derive B_2F_1 plants with 2n = 45-49. B_2F_2 plants with 43 or 44 chromosomes were identified. These were selfed and B_2F_3 with 2n = 44 were selected. In those instances where the B_2F_4 did not produce primarily 2n = 44 progeny, selfing was continued. A total of 30 random putative disomic additions were recovered. DNA extracts were made of each and RFLP markers that were associated with the target chromosomes by Marais and Marais (2003) were used to screen for the presence of chromosomes $2J_1^d$ (PSR666), $3J_1^d$ (PSR931), $4J_1^d$ (PSR921), $5J_1^d$ (PSR628) and $7J_1^d$ /$7J_2^d$ (PSR129) employing methodology outlined by the authors. In this manner disomic additions of $3J_1^d$, $5J_1^d$ and $7J_2^d$ were identified. (ii) B_1F_1 hybrids (97M1) that were confirmed to have either of chromosomes $2J_1^d$ or $4J_1^d$ were used as male parents in RFLP marker-aided backcrosses to 'Rex'. A disomic addition of $2J_1^d$ was selected from the B_2F_3 whereas a disomic addition $4J_1^d$ was selected from the B_3F_3. A $7J_1^d$ addition line could not be found among the 30 random additions. The group 7 chromosomes were not conclusively shown to influence salt tolerance (Marais and Marais, 2003) and if they did, they probably had the smallest effect. It was therefore decided not to continue searching for this addition. C-banding Marais and Marais (2003) was done with each addition line to confirm the presence of the added *Thinopyrum* target chromosome. The putative chromosome $3J_1^d$ addition, 'Rex' triticale and *Th. distichum* were also tested for the presence of EST-5 isozyme variations. Esterase-5 isozymes were extracted and separated by isoelectric focusing as described by Ainsworth *et al.* (1984).

Development of SCAR-markers for *Thinopyrum* chromosome regions homoeologous to mapped wheat cDNA loci

In an initial attempt to develop chromosome specific markers from RFLPs, mapped (diagnostic) wheat cDNA loci were used as basis for the detection of homoeoloci in *Th. distichum*. The cDNA probes and their sequences were kindly supplied by Dr MD Gale (John Innes Centre, Colney, Norwich NR4 7UJ, UK) and included: PSR934 (detects the wheat 2L arms), PSR666 (2S), PSR931 (3L), PSR305 (3S), PSR163 (4L), PSR115 (5L), PSR360 (5L), PSR628 (5S), PSR929 (5S), PSR129 (7L) and PSR160 (7S). In each case software programme BioEdit (Hall, 1999) was used to devise forward and reverse primers based on the cDNA sequence. The primers were then used to amplify bands in a set of genotypes that will be referred to as 'panel 1'. Panel 1 included: *Th. distichum*, 'Rex' triticale, the primary hybrid, 96M2 (= *Th. distichum*/triticale), the primary hybrid 95M1 (= *Th. distichum*/tetraploid 'Henoch' rye), 'Henoch' rye and a set of fifteen F_1: *Th. distichum*/tetraploid rye//diploid rye hybrid clones with known *Thinopyrum* chromosomes (identified by C-banding; Marais and Marais, 2003). DNA was isolated according to Doyle and Doyle (1990). In order to identify amplicons that were associated with the target chromosome and homoeologous to the particular cDNA sequence, the PCR products were probed with the appropriate cDNA sequence following methodology outlined in the DIG Application Manual for Filter Hybridization (Roche Diagnostics GmbH, Roche Molecular Biochemicals, 68298 Mannheim, Germany). If a putative marker for a homoeolocus on a critical *Thinopyrum* chromosome was detected, the relevant fragment was cut from the gel, cloned and sequenced. A new (refined) set of primers was designed and tested on the genotype panel.

Development of SCAR-markers from *Thinopyrum*-specific RAPD fragments

Fifteen RAPD primer sets (OPE, -F, -H, -M, -N, -O, -P, -Q, -R, -S, -T, -U, -V, -W and –X; each consisting of 20 primers), obtained from Operon Technologies ™, Alimede, California, USA were employed. A 25µl PCR mix containing 50ng template DNA, 0.4µM primer, 0.2mM dNTPs (Bioline Ltd, London), 1X reaction buffer (Bioline), 2.5mM $MgCl_2$ and 0.02U Taq polymerase (Bioline) was prepared and the amplification performed in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, California, USA). The thermal cycling conditions were: initial denaturation at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing for 1 min at the calculated annealing temperature, extension at 72°C for 2 min. Final extension was done at 72°C for 5 min. PCR products were separated at constant voltage in a 2% agarose gel containing 0.5µg/mL of ethidium bromide in 1X TBE.

The plant material used for RAPD analysis (panel 2) consisted of *Th. distichum*, 'Rex' triticale, the F_1 hybrid 96M2 and the following monosomic addition plants and disomic addition lines for *Thinopyrum* chromosomes: (a) A plant with 2n = 44 chromosomes including single copies of $2J_1^d$ and $3J_1^d$, (b) disomic addition line $3J_1^d$ (2n = 44), (c) disomic addition line $3J_1^dL$ (2n = 42 + 2t), (d) a

monosomic addition $4J_1^d$ plant (2n = 43), (e) disomic addition line $5J_1^d$ (2n = 44) and (f) disomic addition line $7J_2^d$ (2n = 44). Diagnostic RAPD fragments were cloned, sequenced and the information used to devise longer SCAR primers. SCARs were amplified in an optimized 25µl reaction mixture generally containing 50-100ng template DNA, 0.2µM of each primer, 0.2mM dNTPs, 2.5mM $MgCl_2$ and 0.04U Taq polymerase. Cycling conditions (35 cycles) were the same as for RAPDs. PCR products were separated on 2% agarose gels. All the PCR-markers were then tested on a third panel consisting of 43 genotypes. The same material was used to provide plants with combinations of the critical chromosomes for salt tolerance tests. Panel 3 included (a) the disomic additions for $2J_1^d$, $3J_1^d$, $4J_1^d$, $5J_1^d$ and $7J_2^d$, (b) 19 F_1 of crosses between disomic or monosomic single chromosome addition lines, (c) eight B_2F_2: *Th. distichum*/ 3*triticale plants (having the *Triticum* cytoplasm and generally 43 – 48 chromosomes) and (d) eleven B_1F_2: *Th. distichum*/ 2*triticale plants (having the *Thinopyrum* cytoplasm and generally 51-56 chromosomes).

Cloning, sequencing, primer design and synthesis

Polymorphic fragments were recovered using a gel extraction kit (Qiagen, GmbH, Germany) and ligated in pGEM^R-T easy vector (Promega, Madison, Wisconsin, USA). DH5ά competent cells were transformed with the ligation products using ampicillin /X-gal/IPTG for blue-white colony screening. DNA inserts in positive clones were checked for size (PCR) and sequenced with an Abi Prism 3100 automated sequencer using T7 and SP6 primers. SCAR primer pairs were designed using BioEdit (Hall, 1999) and synthesized by Inqaba Biotechnical Industries Ltd, Hatfield, South Africa.

Salt tolerance of secondary hybrids with combinations of critical chromosomes

Twenty-seven plants with added critical chromosomes (A-genotypes in Table 1) were selected from panel 3 and used for salt tolerance testing. Since $3J_1^d$ followed by $4J_1^d$ and $5J_1^d$ were believed to be the most important chromosomes, emphasis was placed on identifying plants that had $3J_1^d$ in combination with $4J_1^d$ or $5J_1^d$ or which had the combination of $3J_1^d$, $4J_1^d$ and $5J_1^d$. In addition combinations involving the minor $2J_1^d$ or $7J_2^d$ chromosomes or $3J_1^d$ telosome were also included. The controls consisted of the disomic addition lines of chromosomes $3J_1^d$, $3J_1^d$ L, $4J_1^d$, $5J_1^d$ and $7J_2^d$ as well as 'Henoch' (*Secale cereale*), 'Orania' (*Triticum turgidum* ssp durum), 'Rex' (triticale), the F_1: *Th. distichum*/tetraploid rye (95M1), the F_1: *Th. distichum*/ triticale (96M2), *Th. distichum* and a putative $3J_1^d$L translocation to an unknown triticale chromosome. Since the primary hybrids 95M1 and 96M2 as well as some of the secondary derivatives were sterile (particularly those with the *Thinopyrum* cytoplasm), all genotypes were first raised in a greenhouse and then cloned to provide material for salt tests. The total set of 102 plants thus derived (Table 1) were divided into three replicates and plants within replicates were randomly allocated to pots. As is evident from Table 1, two plants were planted per replicate of some genotypes.

The salt tolerance evaluation was done during July to October 2006 in a cooled (12°C/18°C) greenhouse at Welgevallen, Stellenbosch. Two plants were planted per 2L plastic pot filled to four-fifths with coarse sand. Three 8 mm diameter holes were drilled in the side of each pot, approximately three centimetres from the bottom to ensure proper draining. Clones were watered with a standard nutrient solution while establishing. The nutrient solution was made up of 164 g Sol-u-fert T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2 g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa) and 77 ml potassium nitrate in 100 L water. Salt treatment commenced prior to booting. Different salt treatments were prepared by solubilizing equal amounts of NaCl and $CaCl_2$ in standard nutrient solution. Salt levels were raised by 4 dSm^{-1} on alternate days until 38 dSm^{-1} was reached. Each pot was watered daily with 500 ml of the appropriate salt solution. When the maximum salt level was reached each pot received 250 ml of the 38 dSm^{-1} solution per day until the conclusion of the experiment. The experiment was stopped and the plants visually scored when 75-80% of the control 'Rex' triticale plants had died and the remainder were clearly dying. All plants of all the replications of a particular genotype/ chromosome combination were considered together to arrive at a single visual rating for each control genotype or combination of critical chromosomes. The scores given varied from 0 (least tolerant) – 10 (most tolerant) and the system of scoring is explained in Table 1.

RESULTS

Development of addition lines

Backcross derivatives that received the *Triticum* cytoplasm were phenotypically similar to those with the *Thinopyrum* cytoplasm yet had considerably better fertility and seed quality. The fertility of hybrids with the *Thinopyrum* cytoplasm decreased in subsequent selfed generations and chromosome aberrations appeared to accumulate, it was therefore decided to develop additions with the *Triticum* cytoplasm only. When screened with RFLPs, the 30 random addition lines were found to include two $3J_1^d$ additions, four $4J_2^d$ additions, one $5J_1^d$ addition and five $7J_2^d$ additions. Three addition lines ($3J_1^d$, $5J_1^d$ and $7J_2^d$) that regularly produced 2n = 44 progeny were chosen for further characterization. In each case C-banding was done (Fig. 1) to confirm that the correct chromosome as identified by Marais and Marais (2003)

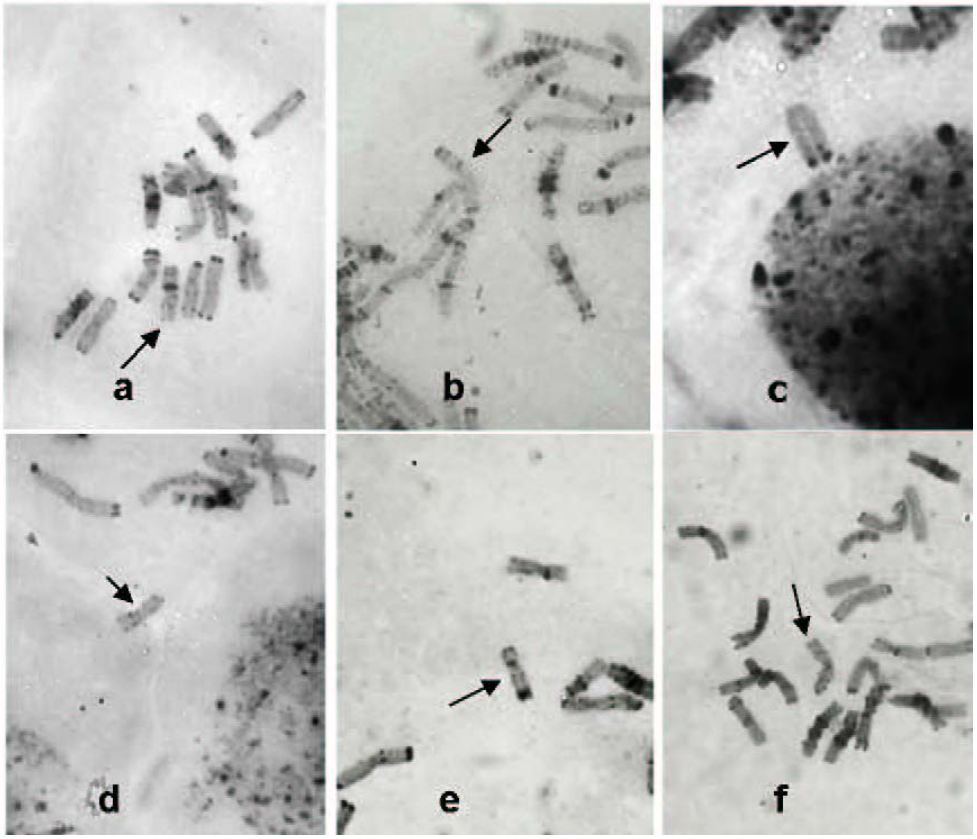

Fig. 1. C-banded mitotic metaphase cells showing the added *Thinopyrum distichum* chromosome in each of six triticale disomic addition lines. The chromosomes (arrowed) are: (a) $2J_1^d$, (b) $3J_1^d$, (c) $3J_1^dL$, (d) $4J_1^d$, (e) $5J_1^d$ and (f) $7J_2^d$.

had been transferred. Addition $3J_1^d$ produced an *Est-5* polymorphism that is also present in *Th. distichum*. Addition $3J_1^d$ was subsequently used in an attempt to produce centric break and fusion (Robertsonian) translocations between $3J_1^d$ and triticale group 3 homoeologues through double monosomy (unpublished results). In the course of these attempts a plant with a $3J_1^dL$ telosome (Fig. 1) was recovered and among its progeny a disomic addition (2n = 42 + 2t) could be identified. Disomic $2J_1^d$ and $4J_1^d$ additions were derived through marker-aided backcrosses to ''Rex'' and both additions were then confirmed by C-banding (Fig. 1).

Conversion of RFLP markers

A total of eleven anonymous cDNA loci were evaluated with the purpose to develop chromosome-specific SCAR markers for the *Thinopyrum* addition chromosomes. However, the success rate was very low, primarily because the presence of triticale and *Thinopyrum* homoeoloci confounded the sequencing data making it difficult to detect polymorphic sequences that could be used as primers. As a result it was possible to amplify and clone chromosome specific fragments for only three of the loci.

The RFLP probe, PSR931, hybridizes to the long arms of the wheat homoeologous group 3 chromosomes. Three orthogolous SCARs (UST5-$3J_1^dL$, UST6-$3J_1^dL$ and UST7-$3J_1^dL$) were derived (Table 2) and confirmed (panel 1) to be specific for chromosome $3J_1^d$. The three markers appear to detect the same locus. Using PSR360 (specific for the wheat group 5L arms) sequence information, a diagnostic primer set (UST10-$5J_1^dL$) that amplifies a 623 bp portion of the orthologous $5J_1^d$ sequence was derived (Table 2). The PSR129 sequence (wheat group 7L arms) was used to design primer set UST11-$7J_2^dL$ (Table 2), which amplifies a single polymorphic fragment in genotype panel 1 that co-segregated with chromosome $7J_2^d$.

Identification and conversion of RAPD markers

Of the 298 RAPD primers tested on genotype panel 2, 41 showed polymorphism between *Thinopyrum* and triticale and also amplified bands specific to one of the addition chromosomes. Only the most distinct bands were converted to SCARs (Table 2). Two SCAR markers, UST1-$2J_1^d$ and UST2-$2J_1^d$ (Table 2), derived from RAPD fragments, have been reported by Jacobs *et al.* (2004) and were confirmed by the authors to amplify a target

Table 1. Addition and control plants used in salt tolerance tests. The tolerance scores assigned to genotypes and the bases for assigning them are also shown.

Score	Genotypes tested[a,b]			Condition of plants at the completion of the tolerance test
		2n	No. of clones	
0	'Henoch' (*Secale cereale*)	14	6	Plants had died before heading
1	'Orania' (*Triticum turgidum* var durum)	28	6	Plants had died after heading and produced some seeds
2	Addition $5J_1^d$ Addition $3J_1^dL$ A3L7 (2 plants)	44 42+2t 43+t	3 3 3	Plants dead or dying; some degree of greenness left in the stems
3	Addition $4J_1^d$ A2β34 (1 plant) A57 (4 plants)	44 44+a[c] 44, 45	4 1 4	Plants dead or dying; higher degree of greenness in the stems
4	'Rex' Addition $3J_1^d$ Translocation $3J_1^dL$ A2β37 (1 plant) A3L4 (3 plants) A35 (2 plants)	42 44 42 44+a[c] 43+t-44+t 44	6 6 6 3 5 3	Plants dead or dying; 20-25% of ears still green
5	Addition $7J_2^d$ A347 (1 plant)	44 44	3 3	Plants dying; about 50% of ears still green
6	A23 (2 plants) A234 (4 plants)	46 46-48	3 5	About 15% of leaf area still green
7	A2345 (1 plant) A23457 (2 plants)	54 51, 53	1 2	About 25-33% of leaf had died (from the tip inwards)
8	*Th distichum*/ tetraploid rye (95M1)	28	6	Leaves still green except for dead tips; no new growth
9	A235 (1 plant) A345 (3 plants)	53 51-56	2 6	Leaves still green but yellowish at the tips; strong new growth
10	*Th distichum* C_1: *Th distichum*/ triticale (96M2)	28 70	6 6	Leaves were green; strong new growth

[a] Backcross-derived genotypes with added (presumably single copies) *Thinopyrum* chromosomes are indicated with the prefix 'A' immediately followed by an indication of the critical *Thinopyrum* chromosomes it contained. Codes for the critical chromosomes are: 2 = $2J_1^d$; 2β = recombined $2J_1^d$?; 3 = $3J_1^d$; 3L = $3J_1^dL$ arm; 4 = $4J_1^d$; 5 = $5J_1^d$ and 7 = $7J_2^d$. For example, A3L7 had single copies of the $3J_1^dL$ telosome and $7J_2^d$.

[b] The plants in categories 7-10 had more complex additions and contained the *Thinopyrum* cytoplasm. All the plants within categories 1 – 6 had the *Triticum* cytoplasm.

[c] Acrocentric chromosome believed to be a *Thinopyrum* B-chromosome.

sequence specific for $2J_1^d$. Another thirteen RAPD markers were subsequently identified for this chromosome of which OPE-16 and OPX-4 bands were converted to SCAR markers (Table 2). The latter two derived markers (UST3-$2J_1^d$ and UST4-J_1^d) and an unconverted, yet repeatable RAPD (OPR-16) band, co-segregated with the markers of Jacobs *et al.* (2004) in test panel 3 (43 plants). However, the UST4-J_1^d polymorphic band was not amplified in three of the panel 3 plants. The three plants were derived from the same cross and the most likely explanation is that locus *Xust4-*J_1^d was lost as a result of recombination with $2J_2^d$ as C-banding showed that a complete $4J_1^d$ was present. Two of the latter plants (A2ß34 and A2ß37 in Table 1) had an acrocentric chromosome present and were also included in the salt tolerance tests. The C-band pattern of the acrocentric chromosome was unlike that of any of the *Thinopyrum* chromosomes and it appeared to be a B-chromosome.

Seven RAPD markers could be associated with chromosome $3J_1^d$. The availability of a $3J_1^dL$ telosome made it possible to assign the markers to chromosome arms. The most distinct polymorphic band among the short arm fragments was converted into a SCAR (UST8-$3J_1^dS$) that detects $3J_1^dS$. UST8-$3J_1^dS$ and the PSR931-derived SCARs co-segregated in test panel 3, however, it also became obvious that UST8-$3J_1^dS$ is not a chromosome specific marker as it also detects $2J_1^d$ and

Table 2. Molecular markers associated with five *Thinopyrum distichum* chromosomes believed to contribute to salt tolerance in this species.

Chro-mosome	Code	SCAR			RAPD		
		Forward and reverse primers (5'-3')	Derived from:	Approxi-mate fragment size (kb)	Code	Primer	Approximate fragment size (kb)
$2J_1^d$	UST1-$2J_1^d$ [a]	CACCTTTCCCTGTTAGGATA CCTGCATCTACTCCAACC	RAPD	0.974	OPK-6	CACCTTTCCC	1.060
	UST2-$2J_1^d$ [a]	TGCTCAATGAAACAGGAGACTG TTGAAACTTCCCTCCTGCG	RAPD	0.597	OPK-17	CCCAGCTGTG	0.652
	UST3-$2J_1^d$	GGTGACTGTGTGGGATGTAC GGTGACTGTGGTGAAGAAAAGC	RAPD	0.433	OPE-16	GGTGACTGTG	0.433
	UST4-$2J_1^d$	CCGCTACCGACTACTACAG CCGCTACCGAACATGATCTC	RAPD	0.636	OPX-4	CCGCTACCGA	0.636
					OPR-16	CTCTGCGCGT	1.000
$3J_1^d$ L	UST5-$3J_1^d$L	TTAGGATTTGTCCACTTGATTG GTTGGTGCTTGGTACCTTAAC	PSR931	0.849			
	UST6-$3J_1^d$L	TCACTCAATGGTGTTGTGG CGACTTCATTATCGGCAAC	PSR931	0.734			
	UST7-$3J_1^d$L	GTTCGATAGCTCATCATAGCAG TTCTAGTGGTTGCAATTGAAGT	PSR931	0.705			
$2J_1^d$, $3J_1^d$ S and $4J_1^d$	UST8-$3J_1^d$S	GTCTTGCGGAACGAGTAAAGAG GTCTTGCGGATGTGAGGAAGTC	RAPD	1.600	OPM-9	GTCTTGCGGA	1.600
$4J_1^d$	UST9-$4J_1^d$	GGAGCCTCAGGATCATCATGC GGAGCCTCAGCAATGAATGGA	RAPD	0.949	OPX-11	GGAGCCTCAG	0.949
	UST15-$4J_1^d$	GAGACGCACAGGGGACCG GAGACGCACACACACGTACA	RAPD	0.587	OPN-6	GAGACGCACA	0.587
$5J_1^d$ L	UST10-$5J_1^d$L	GATGAGAACAGATGGTTACAGAC	PSR360	0.623			
$5J_1^d$	UST13-$5J_1^d$	GAACTCCATGTTAAGAGCTGATGA	RAPD	0.728	OPU-14	TGGGTCCCTC	0.728
	UST14-$5J_1^d$	TGGGTCCCTCAAGCTACGTGT TGGGTCCCTCCAACCCCCC GAAGCCAGCCGAAGAGATCTC GAAGCCAGCCCTTGGGGGT	RAPD	0.505	OPV-7	GAAGCCAGCC	0.505
$7J_2^d$L	UST11-$7J_2^d$L	CTTTCAGGCGTTTCCCGTT GATCTGCTGCAGGTCACCA	PSR129	0.325			
$7J_2^d$	UST12-$7J_2^d$	CAGAGGTCCCATCCACCATTC TCTATCAGGGCAATGTTGGTGAAC	RAPD	0.325	OPS-3	CAGAGGTCCC	

[a]Developed by Jacobs *et al.* (2004)

$4J_1^d$. The *Est-5* polymorphism can serve as a further long arm marker. The availability of confirmed markers for both arms made it possible to initiate an attempt to derive Robertsonian translocations involving $3J_1^d$ and homoeologous group 3 triticale chromosomes.

Two of the more repeatable $4J_1^d$ polymorphisms (OPN-6, OPX-11) have been converted into SCAR markers (UST15-$4J_1^d$ and UST9-$4J_1^d$ in Table 2) and confirmed on test panel 2. Two of the $5J_1^d$ fragments (OPU-14 and OPV-7) were converted into SCAR markers UST13-$5J_1^d$ and UST14-$5J_1^d$, respectively. The two markers were confirmed using test panel 2 and also co-segregated with the PSR360-derived SCAR in test panel 3. A $7J_2^d$ diagnostic RAPD fragment produced by OPS-3 has been converted into SCAR marker UST12-$7J_2^d$ which could be confirmed using test panel 2 and also co-segregated with the PSR129-derived SCAR (UST11-$7J_2^d$) in test panel 3.

Identification and evaluation for salt tolerance of plants with various combinations of added critical chromosomes

The results obtained following evaluation of all the markers on panel 3 allowed for the identification of a series of triticale plants with various combinations of added target chromosomes, in particular chromosomes $3J_1^d$, $4J_1^d$ and $5J_1^d$. These plants were used in an attempt to confirm the effect of the target chromosomes in enhancing salt tolerance of triticale. The results obtained upon completion of the salt screening are summarized in Table 1.

DISCUSSION

The salt tolerance test employed high levels of salt until the control plants had died and was therefore designed to detect major rather than subtle differences. Under these

test conditions single chromosome addition lines did not appear to differ in salt tolerance from the control, 'Rex'. It therefore appears unlikely that transfer of a single chromosome, or parts thereof, would result in an economically useful level of tolerance. The tested combinations that involved two chromosomes at a time (Table 1) included A23, A3L4, A34, A35, A3L7, A37 and A57 (as is explained in Table 1, the numbers in the genotype codes have specific meaning: 2 = $2J_1^d$; 2β = $2J_1^d$ recombined?; 3 = $3J_1^d$; 3L = $3J_1^d$L; 4 = $4J_1^d$; 5 = $5J_1^d$ and 7 = $7J_2^d$). Of these, only the A23 combination appeared more salt tolerant than 'Rex'. Combinations A2β34 and A2β37 appeared less salt tolerant than A23 suggesting that $2J_1^d$ genes involved in salt tolerance may have been lost as a result of recombination with $2J_2^d$. However, this needs to be confirmed. The data did not allow for comparison of the effect of $3J_1^d$L versus the complete $3J_1^d$. The remaining combinations of three chromosomes at a time were A234, A235, A345 and A347. Of these, A235 and A345 appeared to produce very comparable and potentially useful tolerance (score = 9). Their tolerance levels approximated those of the primary *Th. distichum*/triticale amphiploid and *Th. distichum* (score = 10), and similarly, they showed strong new shoot development at the conclusion of the experiment. Combinations A2345 and A23457 were given a score of 7. While the possibility of negative interactions cannot be ruled out it is possible that their lower score is simply due to experimental variation. The results nonetheless suggest that combinations A235 and A345 were the most effective. Combinations that involved chromosome $7J_2^d$ (A3L7, A57, A2β37, A347 and A23457) did not suggest a notable effect for this chromosome. Obviously, the conclusions drawn here regarding interactions among the critical chromosomes may not apply to all situations and the genetic background of the recipient triticale genotype might also be important. Furthermore, the complexity and arrangement of the gene complexes on the target chromosomes are not known, neither can it be predicted to what extent the target chromosomes or sections thereof can substitute for homoeologous regions in triticale. Therefore, at least during the initial stages of transfer it would be best to simultaneously target critical chromosomes $2J_1^d$, $3J_1^d$, $4J_1^d$ and $5J_1^d$.

Different transfer strategies to develop salt tolerant plants are now being attempted and include: (1) Systematic development of Robertsonian translocations between *Thinopyrum* target chromosomes and homoeologous triticale A-, B- or R-genome chromosomes employing double monosomy. The condition is achieved through crosses of the respective *Thinopyrum* addition lines with homoeologous triticale ('Rhino') monosomics or substitution lines (AJ. Lukaszewski, University of California, USA). (2) Development of plants with 2n = 42, $AABBJ_{12}^dJ_{12}^d$, where J_{12}^d will be a balanced set of *Thinopyrum* J_1^d and J_2^d chromosomes among which the primary determinants of salt tolerance. (3) Development of plants with 2n = 56, $AABBRRJ_{12}^dJ_{12}^d$ chromosomes. In deriving the latter two types of plant, use is being made of pivotal genotypes with chromosome compositions similar to those targeted. These are being crossed with secondary hybrids having the target chromosomes. Marker-assisted identification of appropriate progeny for continued crossing to the pivotal genotypes is being employed in an attempt to derive salt-tolerant plants of the desired types.

Since a high level of homoeologous recombination occurs between the chromosomes of the J_1^d and J_2^d genomes when they are in the haploid state, the association between genes being introduced and a SCAR marker may be lost as a result of J_1^d – J_2^d recombination. Similarly, translocations involving triticale chromosomes may result in loss of association between marker loci and salt tolerance genes. The addition lines are therefore also being used to find large numbers of additional AFLP markers for the critical chromosomes. It is also clear that regular and stringent testing of backcross derivatives for salt tolerance will need to form part of the introgression process. Intermediates produced during transfer will furthermore be used in association analyses to link sub regions (haplotypes) of the critical chromosomes with salt tolerance. Thus, it is envisaged that refinement of marker assisted selection and improved definition of critical (target) chromosome sub regions could proceed in parallel with the attempted development of commercially useful plant types.

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