Introgression of wheat chromosome 2D or 5D into tritordeum leads to free-threshing habit

S.G. Atienza, A.C. Martín, and A. Martín

Abstract: Hexaploid tritordeum is the amphiploid derived from the cross between the diploid wild barley *Hordeum chilense* and durum wheat. The non-free-threshing habit is a constraint to this species becoming a new crop. Three tritordeum lines (HT374, HT376, and HT382) showing the free-threshing habit were selected from crosses between tritordeum and bread wheat. All three lines were euploids, as revealed by mitotic chromosome counting. Genomic in situ hybridization analysis made it possible to distinguish differences among these lines. While the line HT382 carries only 10 chromosomes from *H. chilense*, the lines HT374 and HT376 have 12. These results suggest that HT382 is a double chromosome substitution line between *H. chilense* and the wheat D genome, while HT374 and HT376 each have one pair of *H. chilense* (H^{ch}) chromosomes substituted by wheat D chromosomes. Molecular characterization revealed that HT382 is a 1D/($1H^{ch}$), 2D/($2H^{ch}$) chromosome substitution line, whereas HT374 and HT376 have 5D/($5H^{ch}$) substitutions. On the basis of previous knowledge, it seems that the absence of chromosome $2H^{ch}$ or $5H^{ch}$ is more important for producing the free-threshing habit than the presence of chromosome 2D or 5D, while chromosome $1H^{ch}$ seems to be unrelated to the trait. These free-threshing tritordeum lines constitute an important advance in the tritordeum breeding program.

Key words: Hordeum chilense, threshability, amphiploid.

Résumé : Le tritordeum hexaploïde est un amphidiploïde obtenu en croisant l'orge sauvage *Hordeum chilense* et le blé dur. Les grains vêtus obtenus lors de la récolte de cette espèce constituent une contrainte à sa culture. Trois lignées du tritordeum (HT374, HT376 et HT382) produisant des grains nus ont été sélectionnées à partir de croisements entre le tritordeum et le blé tendre. Des décomptes chromosomiques à la mitose ont montré que ces trois lignées étaient euploïdes. Une analyse GISH a permis d'identifier les différences entre ces lignées. Tandis que la lignée HT382 compte seulement 10 chromosomes du *H. chilense*, les lignées HT374 et HT376 en comptent 12. Ces résultats suggèrent que HT382 serait le fruit d'une double substitution chromosomique entre le génome du *H. chilense* et le génome D du blé, tandis que HT374 et HT376 ont chacune une paire de chromosomes H^{ch} remplacées par ceux du génome D du blé. Une caractérisation moléculaire a révélé une substitution 1D/($1H^{ch}$) et 2D/($2H^{ch}$) chez HT382 tandis que HT374 et HT376 ont toutes deux une substitution 5D/($5H^{ch}$). Sur la base de travaux antérieurs, il semblerait que l'absence des chromosomes $2H^{ch}$ ou $5H^{ch}$ serait plus importante pour la production de grains nus que la présence des chromosomes 2D ou 5D tandis que le chromosome $1H^{ch}$ serait sans lien avec ce caractère. Les lignées du tritordeum à grains nus constituent une avancée importante dans le cadre du programme d'amélioration génétique du tritordeum.

Mots-clés : Hordeum chilense, aptitude au battage, amphiploïde.

[Traduit par la Rédaction]

Introduction

Plant breeders have been trying to develop wheat–barley hybrids since the beginning of the 20th century. However, the first fertile amphiploid, ×*Tritordeum* Asch. et Graeb., was obtained only when *Hordeum chilense* Roem. et Schult. was used in crosses with wheat (Martin and Chapman 1977). The high crossability of *H. chilense* (Bothmer and Jacobsen 1986) is a clear advantage for the transfer of useful genes from this wild barley to other species such as wheat. *Hordeum chilense* is highly polymorphic for traits of interest in cereal breeding, such as endosperm storage proteins (Alvarez et al. 2006; Atienza et al. 2005*a*; Atienza et al. 2000), carotenoid content (Atienza et al. 2004), and resistance to biotic stresses (Martín et al. 1999).

Hexaploid tritordeum ($2n = 6x = 42$; $AABBH^{ch}H^{ch}$) is the amphidiploid derived from the cross between *H. chilense* ($2n = 2x = 14$; $H^{ch}H^{ch}$) and durum wheat, *Triticum turgidum* L. (Thell) ($2n = 4x = 28$; AABB). Tritordeum has the potential to become a new crop (Martín et al. 1999) and, therefore, a breeding program is being conducted at the Institute for Sustainable Agriculture (IAS-CSIC) in Córdoba, Spain, with a dual purpose: to develop a new crop and also to obtain plant materials useful for wheat breeding.

The grain and flour of tritordeums have properties comparable to those of bread wheat (Alvarez et al. 1995), but with higher pigment content (Alvarez et al. 1999; Atienza et al. 2007*a*; Atienza et al. 2005*b*; Ballesteros et al. 2003), so grain production for food and feed would be the main use of tritordeum. All primary tritordeums show a non-free-

Received 25 April 2007. Accepted 29 August 2007. Published on the NRC Research Press Web site at genome.nrc.ca on 20 October 2007.

Corresponding Editor: P. Gustafson.

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 doi:10.1139/G07-081

threshing habit, as is seen in synthetic hexaploid wheat (Kerber and Rowland 1974; Lange and Jochemsen 1992; Villareal et al. 1996), and this is a constraint to the acceptance of tritordeum as an alternative cereal crop.

Several genetic systems have been reported as being associated with the free-threshing habit in hexaploid wheat. First, a polygenic system scattered throughout all 3 genomes and counteracting glume tenacity and rachis brittleness was proposed by MacKey (1966). A second system is related to the major gene or gene complex *Q* (MacKey 1954; Muramatsu 1963). The *Q* gene confers the free-threshing character and square-spike phenotype and pleiotropically affects other traits including glume tenacity and rachis fragility. The *Q* gene is located in the long arm of chromosome 5A and has recently been isolated and characterized (Faris et al. 2003; Simons et al. 2006). A third system related to the free-threshing habit was discovered using synthetic hexaploid wheat. These amphiploids are non-free-threshing (Kerber and Dyck 1969) despite the expected homozygosity for the *Q* factor inherited from the free-threshing tetraploid parent. The *Tg* locus, located on chromosome 2D (Jantasuriyarat et al. 2004; Kerber and Rowland 1974), codes for tenacious glumes and is supposed to inhibit the expression of *Q*. At present it is thought that a recessive *tg* allele as well as a dominant *Q* allele must be present for the expression of the free-threshing character in hexaploid wheat (Kerber and Rowland 1974).

Complete threshability in barley is derived from a completely different genetic system. In contrast to those of other cereals, the glumes of barley are normally fused to the seed, producing hulled kernels, but a few domesticated varieties exist that have naked seeds. This trait is controlled by a single recessive gene, called *nud* (*n*), located on chromosome 7H, which confers complete threshability.

The free-threshing habit has been sought since the beginning of the tritordeum breeding program. Although tough-rachis plants have been identified during the years, they have proved to be aneuploids with either 40 or 41 chromosomes. In addition, these plants have been characterized by low fertility, which may influence rachis toughness.

The development of free-threshing tritordeum lines may be possible through several strategies including selection from spontaneous mutation, mutagenesis, or chromosome manipulation, either by substitution, translocation, or recombination into tritordeum of chromosomes from related species. However, to date, the free-threshing habit has not been achieved via spontaneous mutation nor by mutagenesis.

During the last decade we have developed a crossing program between tritordeum and common wheat to develop D/(H^{ch}) chromosome substitution lines. Lines showing improved threshability have been selected and used in the breeding program, and free-threshing lines showing good fertility and grain filling have now been developed.

In the present work we report the cytogenetic and molecular characterization of 3 D/(H^{ch}) substitution lines of tritordeum showing the free-threshing habit.

Materials and methods

Plant material

An extensive search for the free-threshing habit was conducted in the tritordeum breeding program using lines derived from crosses between tritordeum and common wheat. The lines HT374, HT376, and HT382 were selected as free-threshing and used for cytogenetic and molecular characterizations.

The plant material used in this study for the molecular characterization included *H. chilense* accessions H1 and H7 ($2n = 2x = 14$; $H^{ch}H^{ch}$), *Triticum urartu* Tumanian ex Gandilyan ($2n = 2x = 14$; A^uA^u), *Triticum turgidum* L. subsp. *durum* (Desf.) Husn. 'Yavaros' and 'Langdon' ($2n = 4x = 28$; AABB), and *T. aestivum* L. 'Chinese Spring' ($2n = 6x = 42$; AABBDD). In addition, a set of chromosome addition lines of *H. chilense* H1 in 'Chinese Spring' bread wheat developed by Miller et al. (1981) (kindly supplied by Steve Reader, John Innes Centre, Norwich, UK) was also used. This set of addition lines comprised the following: *T. aestivum* 'Chinese Spring' – *H. chilense* (H1) disomic addition lines A ($4H^{ch}$), B ($5H^{ch}$), C ($6H^{ch}$), and D ($7H^{ch}$) ($2n = 6x + 2 = 44$), and *T. aestivum* 'Chinese Spring' – *H. chilense* (H1) ditelosomic addition lines E ($1H^{ch}$ + t) ($2n = 6x + 1 + t = 44$), F ($2H^{ch}\alpha$), G ($7H^{ch}\alpha$), and H ($7H^{ch}\beta$) ($2n = 6x + 2t = 44$).

We initiated a crossing program between tritordeum and common wheat with the aim of developing free-threshing tritordeums through chromosome substitution. Three tritordeum accessions with improved threshability were derived after 6–7 selfing generations from crosses of (tritordeum × bread wheat) × tritordeum and thus they were expected to be substitution lines involving D and H^{ch} chromosomes.

Cytological observations

For somatic chromosome counting, root tips 1 cm long were collected from germinating seeds and pretreated for 4 h in an aqueous colchicine solution (0.05%) at 25 °C. They were fixed in a freshly prepared 100% ethanol – acetic acid (3:1, *v*/*v*) mixture and stained by the conventional Feulgen technique (Feulgen and Rossenbeck 1924).

Fluorescence in situ hybridization (FISH)

Seeds were germinated on moistened filter paper in Petri dishes at 25 °C in the dark in a growth chamber. Root tips 1 cm long were pretreated for 4 h in a 0.05% colchicine solution at 25 °C and fixed in 100% ethanol – acetic acid (3:1, *v*/*v*) for at least 2 weeks. After this, root tips were stained with 1% (*w*/*v*) acetocarmine for 2 min and meristems were scraped out and squashed in 45% (*v*/*v*) acetic acid. Slides were then frozen in liquid nitrogen and covers slips were removed. Finally, the slides were dried at room temperature and stored at 4 °C until use.

Before hybridization, chromosome preparations were treated with DNase-free RNase (100 µg/mL RNase in 2× SSC) for 2 h at 37 °C, washed twice in 2× SSC for 10 min at room temperature and 2× SSC for 5 min at 37 °C, and subsequently treated with pepsin solution (5 µg/mL in 0.01 mol/L HCl) for 10 min at 37 °C. Slides were then washed twice in 1× PBS for 5 min, post-fixed in 1% formaldehyde solution, 50 mmol/L $MgCl_2$ in PBS for 10 min, washed in 1× PBS for 5 min, dehydrated through 70% and 100% ethanol (3 min each), and air-dried.

Genomic *H. chilense* DNA was labelled by nick translation with biotin-11-dUTP (Roche Corporate, Basel, Switzer-

land) to be used as a probe. The in situ hybridization protocol was that of Cabrera et al. (2002). Biotin-labelled probes were detected with streptavidin–Cy3 conjugates (Sigma, St. Louis, Missouri, USA). Chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole) 339 and mounted in VECTASHIELD® (Vector Laboratories, Burlingame, California, USA). A Leica epifluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) was used to visualize the signals. Images were captured using a SPOT CCD camera and the appropriate SPOT 2.1 software (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA) and processed with PhotoShop 7.0 software (Adobe Systems Inc., San Jose, California, USA).

Molecular characterization

Primer selection

Two batteries of markers were selected to differentially detect the D and H^{ch} chromosomes. For the D genome, the chromosome-specific SSR markers developed by Röder et al. (1998) were chosen. The SSR loci selected were Xgwm337-1D, Xgwm261-2D, Xgwm161-3D, Xgwm194-4D, Xgwm272-5D, Xgwm325-6D, and Xgwm44-7D. The primer sequences corresponding to these loci were reported by Röder et al. (1998).

Barley EST markers developed by Nasuda et al. (2005) were selected from among those previously assigned to *H. chilense* chromosomes by Hagras et al. (2005*a*). The loci selected were k01339-1H^{ch}, k01360-2H^{ch}, k01242-4H^{ch}, k01184-5H^{ch}, k01323-5H^{ch}, and k01062-6H^{ch}. For chromosome 7H^{ch} we used the CAPS marker previously developed by Atienza et al. (2007*b*). The primer sequences corresponding to these loci were reported by Hagras et al. (2005*b*).

DNA extraction and PCR conditions

Genomic DNA was extracted according to the protocol of Murray and Thompson (1980). PCR amplification was performed with 40 ng of genomic DNA as template in a volume of 25 μL using the Biotools DNA polymerase (Biotools, B & M Labs, S.A.) and the recommendations of the supplier.

Amplifications were carried out using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, California, USA). For the SSR markers we followed the conditions described by Röder et al. (1998): 3 min at 94 °C followed by 45 cycles of 1 min at 94 °C, 1 min at the optimal annealing temperature, and 2 min at 72 °C. A final extension step of 10 min at 72 °C was performed. For the barley EST markers we performed an initial denaturation step at 94 °C for 3 min, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR amplification products were resolved by 2% (*w*/*v*) agarose gel and visualized with ethidium bromide.

Results

Attempts to develop free-threshing tritordeums through mutagenesis have to date not been successful (unpublished data). In other cereal amphiploids, the development of substitution lines has proven to be an efficient tool for the improvement of important agronomic traits. For instance, triticale breeding benefited from the selection of the 'Armadillo' lines (Zillinsky 1974), which showed improved fertility due to the chromosome substitution 2D/(2R). Low fertility was, at that time, the major constraint in triticale breeding (Zillinsky 1974) and the production of these lines was an important milestone in the development of triticale as a cereal crop.

Fig. 1. Threshability comparison between free-threshing (A and B) and non-free-threshing (C) tritordeum lines: (A) line HT382, (B) line HT374, and (C) line HT349.

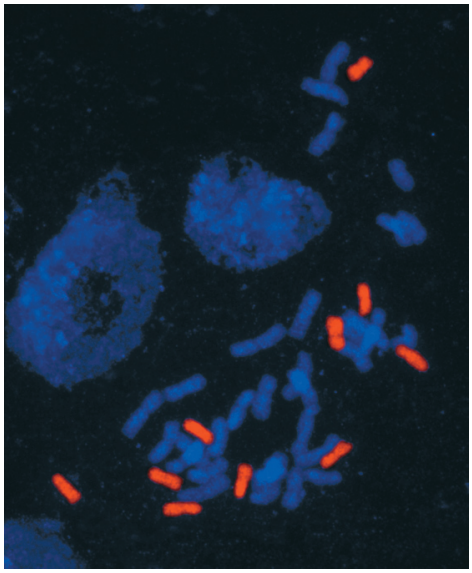
Three tritordeum lines (HT374, HT376, and HT382) were derived from the crossing program between tritordeum and common wheat. These lines represent a considerable advance in tritordeum breeding because of their improved threshability compared with non-free-threshing tritordeums, as shown in Fig. 1. All 3 lines were derived from crosses of (tritordeum × bread wheat) × tritordeum and thus they were expected to be substitution lines involving D and H^{ch} chromosomes.

Cytogenetic characterization

Chromosome counting from somatic preparations revealed that all 3 free-threshing lines were euploid, with 42 chromosomes. Tough-rachis free-threshing lines had been previously detected in our breeding program but they were always aneuploids with either 40 or 41 chromosomes. In addition, they usually showed low fertility, which probably influenced rachis toughness. In contrast, HT374, HT376, and HT382 are fully fertile and therefore are useful for tritordeum breeding.

Although all 3 lines are euploids, the FISH analysis revealed differences among them. Genomic in situ hybridization (GISH) was performed using *H. chilense* genomic DNA as probe (detected with biotin (red), while DAPI staining (blue) shows the wheat chromosomal DNA). The line HT382 was found to have only 10 chromosomes from *H. chilense* (Fig. 2), while HT374 and HT376 have 12 *H. chilense* chromosomes. The *H. chilense* chromosomes display an intense red colour. These findings suggest the existence of a double chromosome substitution between

Fig. 2. GISH using *H. chilense* genomic DNA as probe (detected with biotin, red) in metaphase cells of the free-threshing line HT382. Blue DAPI staining shows the wheat chromosomal DNA. HT382 carries 10 *H. chilense* chromosomes (in red), suggesting this is a double chromosome substitution between *H. chilense* and wheat D chromosomes.



H. chilense and D chromosomes in the line HT382 and a single substitution in the lines HT374 and HT376.

Molecular marker characterizations

The diagnostic bands amplified with the markers k01339 and k01360, corresponding to chromosomes $1H^{ch}$ and $2H^{ch}$, respectively, were missing in the line HT382 (Figs. 3A and 3B). In addition, amplification with chromosome-specific SSR markers revealed that the line HT382 carries chromosomes 1D and 2D (Figs. 3C and 3D).

Taking into account that HT382 only has 10 chromosomes from *H. chilense,* as revealed by FISH, we can conclude that HT382 has a double substitution, 1D/($1H^{ch}$), 2D/($2H^{ch}$). Microsatellite markers have been used previously to distinguish chromosome substitution lines (Korzun et al. 1997; Wu et al. 2006). Similarly, the barley EST markers developed by Nasuda et al. (2005) constitute an efficient tool to detect *H. chilense* chromosomes in a wheat background.

The tritordeum lines HT374 and HT376 were found to have a substitution, 5D/($5H^{ch}$), as revealed by the amplification of the locus Xgwm272-5D and the absence of the loci k01323 and k01184, which correspond to chromosome $5H^{ch}$ (Fig. 4).

Discussion

The *H. chilense* genome is colinear to other Triticeae genomes, including those of bread wheat and *H. vulgare* (Hernández et al. 2001). This colinearity has been further demonstrated by the candidate gene approach (Atienza et al. 2007*b*) and by physical mapping of barley ESTs (Hagras et al. 2005*a*).

From our results, it appears that at least 2 different genetic systems may control the free-threshing habit in tritordeum, since 2 different substitution lines show the same phenotype. The colinearity found among grass genomes suggests that the genetic systems controlling the free-threshing habit in tritordeum may be homoeologous to those described in other Triticeae species (Jantasuriyarat et al. 2004; Kerber and Rowland 1974; MacKey 1954, 1966; Muramatsu 1963; Simons et al. 2006).

The line HT382 carries a double substitution, 1D/($1H^{ch}$), 2D/($2H^{ch}$). Previously, Ballesteros et al. (2003) developed tritordeum lines with the 1D/($1H^{ch}$) substitution to improve bread-making quality by adding the locus *Glu-D1*. However, they did not detect any beneficial effect on threshability from this substitution. Consequently it is likely that the 2D/($2H^{ch}$) substitution is responsible for the free-threshing habit shown by HT382. Nevertheless, we cannot rule out the possibility that the substitution 1D/($1H^{ch}$) may have some effect on the trait.

If we postulate that the substitution 2D/($2H^{ch}$) is alone responsible for the free-threshing habit in the line HT382, then either the absence of chromosome $2H^{ch}$ or the presence of chromosome 2D must control the trait.

The development of synthetic hexaploid wheat allowed the identification of the locus *Tg* from chromosome 2D of *Aegilops tauschii* (Kerber and Rowland 1974). The *Tg* allele codes for tenacious glumes and influences threshability (Jantasuriyarat et al. 2004). Common wheat carries a *tg* allele on chromosome 2D. Consequently, the tritordeum line HT382 carries a *tg* allele from common wheat, while it should lack the homoeologous *Tg* allele from *H. chilense*. Whether it is the presence of *tg* from common wheat or the absence of *Tg* from *H. chilense* that confers the free-threshing habit cannot be decided from the present data. However, octoploid tritordeums ($2n = 8x = 56$; $AABBDDH^{ch}H^{ch}$) show the non-free-threshing habit despite carrying chromosome 2D with the *tg* allele. This suggests that it is the absence of chromosome $2H^{ch}$ that confers the free-threshing habit in HT382, probably because of the absence of a homoeologous *Tg* locus from *H. chilense* that codes for tenacious glumes.

The lines HT374 and HT376 carry the substitution 5D/($5H^{ch}$). As proposed above for the 2D/($2H^{ch}$) substitution, it seems that the absence of $5H^{ch}$ is more critical than the presence of 5D to produce the free-threshing habit, since octoploid tritordeums carrying chromosome 5D are not free-threshing. Thus, a genetic system located on chromosome $5H^{ch}$ must affect the trait.

This genetic system could be homoeologous to the wheat domestication *Q* factor located on chromosome 5A. The *Q* gene confers the square-headed phenotype and the free-threshing character of domesticated hexaploid bread wheat and has been suggested to be the wheat *AP2*-like gene (Faris et al. 2003). This hypothesis has recently been corroborated by Simons et al. (2006), who also confirmed the pleiotropic effects of *Q* on rachis fragility and glume tenacity.

The importance of this locus in wheat leads us to suppose that a homoeologous *q* locus may also be active in *H. chilense* and other related grasses. If this is the case, the presence of the qH^{ch} locus would be responsible for the non-free-threshing habit shown by tritordeum and it may be pleiotropically affecting other loci such as the *tg* locus, as reported in wheat (Simons et al. 2006). In synthetic wheat,

Fig. 3. Molecular marker characterizations of the line HT382. Amplifications correspond to markers k01339-1H^{ch} (A), k01360-2H^{ch} (B), WMS337 1DL (locus Xgwm337-1D) (C), and WMS261 2DS (locus Xgwm261-2D) (D). Lanes: H1, *H. chilense* H1; H7, *H. chilense* H7; Tu, *T. urartu*; T539, *T. turgidum* subsp. *durum* 'Langdon'; T21, *T. aestivum* 'Chinese Spring'; T21-1H^{ch} and T21-2H^{ch}, chromosome addition lines of H1 in 'Chinese Spring' of chromosomes 1H^{ch} and 2H$^{ch}\alpha$, respectively; HT382-1 and HT382-2, HT382.

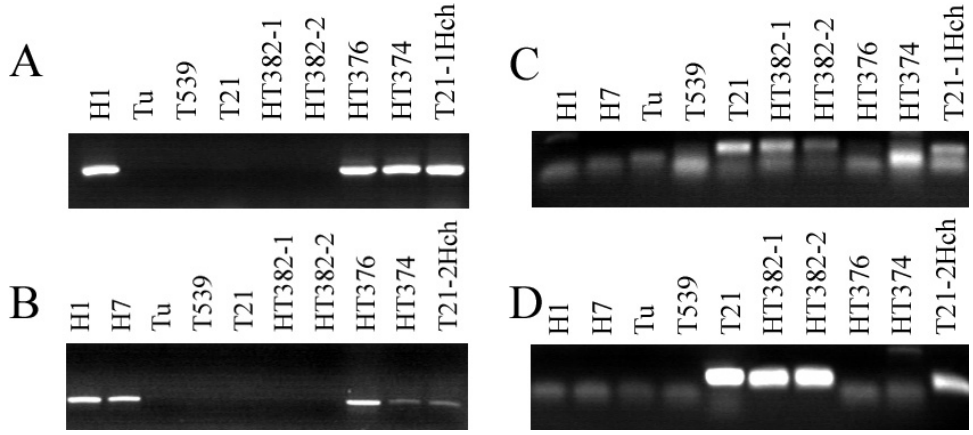
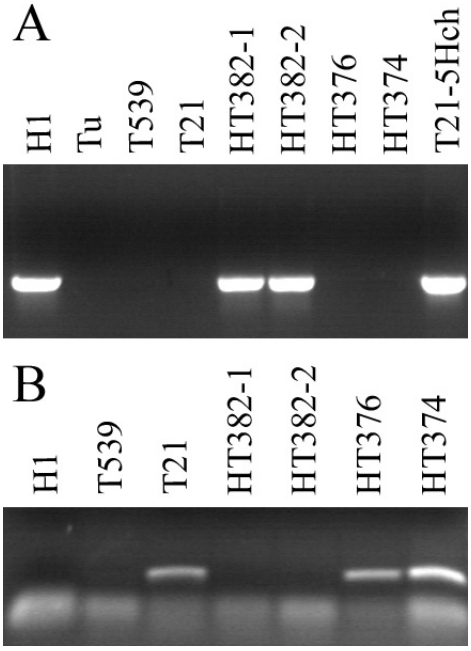


Fig. 4. Molecular marker characterizations of the lines HT374 and HT376. Amplifications A and B correspond, respectively, to markers k01323-5H^{ch} and WMS272 5DL (locus Xgwm272-5D). Lanes are as specified for Fig. 3. T21-5H^{ch} is the chromosome addition line of *H. chilense* H1 in *T. aestivum* 'Chinese Spring' of chromosome 5H^{ch}.



it has been hypothesized that *Tg* is a semidominant gene that interferes with the expression of *Q* on chromosome 5A, producing non-free-threshing plants despite the expected homozygosity for the *Q* factor (Jantasuriyarat et al. 2004; Kerber and Rowland 1974). Nevertheless, it may be possible that a homologous *q* from *A. tauschii* with pleiotropic effects on *Tg* is also active in synthetic wheat. Indeed, the results obtained by Kerber and Rowland (1974) using monosomic and telosomic analysis suggest that chromosome 5D may be related to threshability, although the authors discarded this possibility.

Our data fit with this hypothesis. First, the absence of chromosome 2H^{ch}, where a hypothetical *Tg* locus would be located, leads to free-threshing plants despite the presence of the *qH^{ch}* locus on chromosome 5H^{ch}. Second, the absence of the *qH^{ch}* locus also leads to the free-threshing phenotype despite the presence of chromosome 2H^{ch}. Since the *Q* factor pleiotropically affects the *Tg* locus in bread wheat, it is logical to expect that the *qH^{ch}* locus would also affect the homoeologous *Tg* from *H. chilense*. Although the absence of *qH^{ch}* is at present the best explanation for the free-threshing habit shown by the tritordeum lines HT374 and HT376, interactions of this locus with other loci on the same chromosome cannot be ruled out without further experiments.

If it is true that the *qH^{ch}* locus is responsible for the non-free-threshing habit shown by the tritordeum lines, theoretically it would be possible to obtain free-threshing plants through gene silencing. However, Simons et al. (2006) found that *Q* and *q* alleles differ both in structure and at the level of transcription. This finding may be associated with the conclusions reported by Muramatsu (1963), who showed that *q* has the same effect as *Q*, but to a lesser degree. If the *qH^{ch}* gene functions similarly, it might be possible to obtain free-threshing tritordeums through increased expression.

In conclusion, until now the non-free-threshing habit has been considered an important constraint to tritordeum cultivation. Thus the development of free-threshing lines through chromosome substitution represents a very significant advance in the development of tritordeum as an alternative cereal crop.

Acknowledgments

This paper is dedicated to the memory of Juan Ballesteros. We acknowledge Dr. P. Lazzeri for the critical review of this manuscript and C.M. Ramírez for technical assistance. S.G. Atienza acknowledges financial support from the "Ramón y Cajal Program" from the Spanish Ministry of Education and Science (MEC). A.C. Martín acknowledges the CSIC for a predoctoral fellowship. This research was financed by project AGL2005-01381 from MEC and the Fondo Europeo de Desarrollo Regional (FEDER).

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