

Development of cost-effective *Hordeum chilense* DNA markers: molecular aids for marker-assisted cereal breeding

P. HERNÁNDEZ¹, G. DORADO², M.C. RAMÍREZ¹, D.A. LAURIE³, J.W. SNAPE³ and A. MARTÍN¹

¹ Instituto de Agricultura Sostenible (CSIC), Córdoba, Spain

² Departamento de Bioquímica y Biología Molecular, Campus de Rabanales, Córdoba, Spain

³ Crop Genetics Department, John Innes Centre, Norwich, UK

Hernández, P., Dorado, G., Ramírez, M. C., Laurie, D. A., Snape, J. W. and Martín, A. 2003. Development of cost-effective *Hordeum chilense* DNA markers: molecular aids for marker-assisted cereal breeding. — *Hereditas* 138: 54–58. Lund, Sweden. ISSN 0018-0661. Received April 2, 2002. Accepted February 20, 2003

Hordeum chilense is a potential source of useful genes for wheat breeding. The use of this wild species to increase genetic variation in wheat will be greatly facilitated by marker-assisted introgression. In recent years, the search for the most suitable DNA marker system for tagging *H. chilense* genomic regions in a wheat background has led to the development of RAPD and SCAR markers for this species. RAPDs represent an easy way of quickly generating suitable introgression markers, but their use is limited in heterogeneous wheat genetic backgrounds. SCARs are more specific assays, suitable for automation or multiplexing. Direct sequencing of RAPD products is a cost-effective approach that reduces labour and costs for SCAR development. The use of SSR and STS primers originally developed for wheat and barley are additional sources of genetic markers. Practical applications of the different marker approaches for obtaining derived introgression products are described.

Pilar Hernández, Instituto de Agricultura Sostenible (CSIC), Apdo. 4084, ES-14080 Córdoba, Spain. E-mail gelhemop@uco.es

INTRODUCTION

The wild South American barley species *Hordeum chilense* Roem. et Schult. contains beneficial genes for biotic and abiotic stress resistance, as well as important quality traits such as carotene content and seed storage proteins, many of which are expressed in a wheat background (MARTÍN et al. 1998). Tritordeum, the barley-wheat amphiploid, is the basic genetic material for using *H. chilense* genetic variability in wheat breeding (MARTÍN et al. 1996). The use of this wild species to increase genetic variation in wheat will be greatly facilitated by marker-assisted introgression. To do this, molecular markers that enable tracking of *H. chilense* chromatin in a wheat background are needed.

The practical use of genetic markers is nowadays predominantly performed through the PCR technique. The original PCR methodology first described by Khorana's group (KLEPPE et al. 1971; PANET and KHORANA 1974) and fourteen years later re-discovered and popularized by Mullis et al. (SAIKI et al. 1985, 1986; MULLIS and FALOONA 1987) is conditioned by a priori knowledge of the nucleotide sequences flanking the loci. For a wild species like *H. chilense* where no sequence data are available, this implies the construction of a genomic library, its screening for potentially polymorphic markers and the sequencing of a high number of clones. As this is not economically feasible for a wild species with

limited direct agricultural use, different approaches have been undertaken in a search for suitable and cost-effective markers.

MATERIALS AND METHODS

Plant material

H. chilense lines H1 and H7, *H. vulgare* cv. 'Betzes' and bread wheat cv. 'Chinese Spring' were used for initial RAPD screening and SSR and STS marker transferability studies. *H. chilense* lines H1 and H7 belong to two very distinct ecophysiological and molecular groups (MARTÍN et al. 1998; VAZ PATTO et al. 2001). To assign markers to chromosomes, six wheat (cv. 'Chinese Spring')/*H. chilense* accession H1 addition lines (MILLER et al. 1982) including a monotelodisomic 1 H^{ch}S addition, a ditelosomic addition for 2 H^{ch} alpha arm and disomic addition lines for chromosomes 4 H^{ch}, 5 H^{ch}, 6 H^{ch} and 7 H^{ch} were used. For comparison with *H. vulgare* chromosome locations, six *T. aestivum* (cv. 'Chinese Spring')-*H. vulgare* (cv. 'Betzes') disomic addition lines for chromosomes 2 H^v, 3 H^v, 4 H^v, 5 H^v, 6 H^v and 7 H^v (ISLAM et al. 1978) were used.

DNA extraction

DNA was extracted from young frozen leaf tissue using the CTAB method of MURRAY and THOMPSON (1980) with modifications. The concentration of

each sample was estimated by comparing their band intensities with lambda DNA controls of known concentrations after ethidium bromide staining of 0.8 % agarose gels subjected to electrophoresis.

PCR amplification and marker analysis

Methods for RAPD analysis and SCAR development are described by HERNÁNDEZ et al. 1996, 1999. SSR and STS analysis are also detailed elsewhere (HERNÁNDEZ et al. 2002b,c).

RESULTS AND DISCUSSION

Approaches for the development of cost-effective molecular markers

The practical application of molecular markers in a breeding programme requires simple and economic methods due to the high numbers of individuals that need to be characterized every generation. The advent of PCR-based molecular markers has made molecular tools accessible for breeder use. However, when the breeder needs to use molecular tools for a wild species like *H. chilense*, two more constraints are usually faced: a lack of sequence information available for the wild species genome, and strong economic limitations to marker development when working with a non-crop species. For these reasons, RAPDs were the first DNA molecular markers to be developed for *H. chilense* (see Fig. 1a for a *H. chilense* RAPD amplification profile example).

RAPDs represent an easy way of quickly generating markers suitable for introgression (HERNÁNDEZ et al. 1995; PEIL et al. 1997), but their use is limited when the wheat genetic background changes. SCARs

(PARAN and MICHELMORE 1993) are more specific assays, suitable for automation or multiplexing. To reduce labour and costs for SCAR development, direct sequencing of RAPD products is the preferred approach for their development (HERNÁNDEZ et al. 1999). This approach is based on a modification of the standard RAPD protocol, consisting of the use of decamer primers in pairwise combinations, instead of a single decamer (WELSH and McCLELLAND 1991). In this way, mixtures of RAPD amplification products flanked by the same or by two different primers are obtained. After a simple selection of RAPD products flanked by two different RAPD primers (HERNÁNDEZ et al. 1999), products can be directly sequenced using dye-terminator cycle sequencing, thus avoiding the cost and time-consuming cloning step usually required for SCAR development (PARAN and MICHELMORE 1993).

There is a major difference in the nature of the sequence amplified by SCARs obtained by cloning or by direct sequencing of RAPD products. When cloning the selected RAPD fragment, the original RAPD polymorphism can be reproduced. When directly sequencing the selected RAPD markers, the sequence within the RAPD fragment is used for priming instead of the immediate 3' extensions including the original primers. Such an amplification of sequences internal to the original RAPD maintains the polymorphism, due to significant sequence divergence between the wheat and *H. chilense* genomes. SCAR primer design by cloning and by direct sequencing of RAPD products is illustrated in Fig. 2.

Reamplification of non-targeted bands is the main problem for the development of SCARs by direct sequencing of RAPD products because it leads to

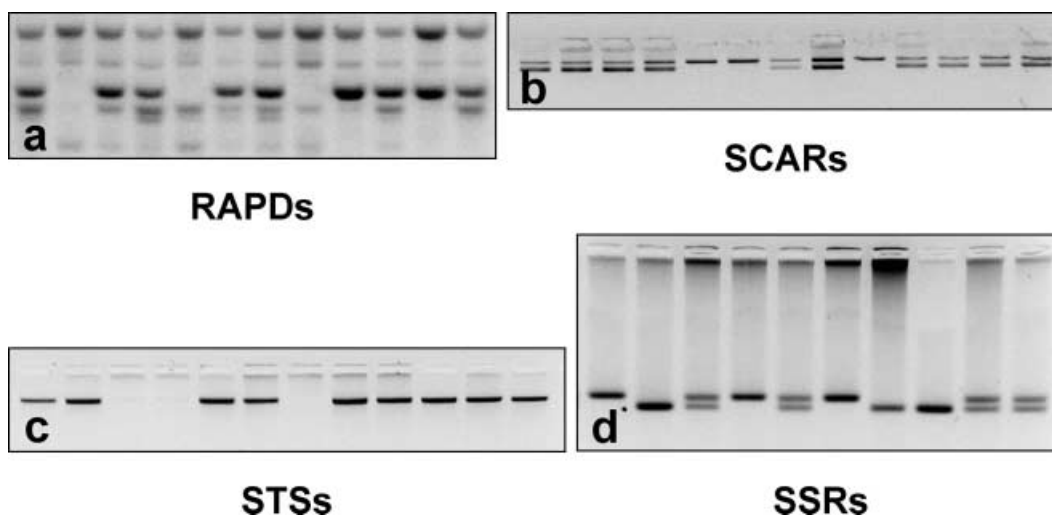


Fig. 1. *H. chilense* F2 H1 × H7 segregation of different types of PCR-based molecular markers. Dominant RAPD (a), SCAR (b) and STS (c) markers and (d) codominant SSR marker.

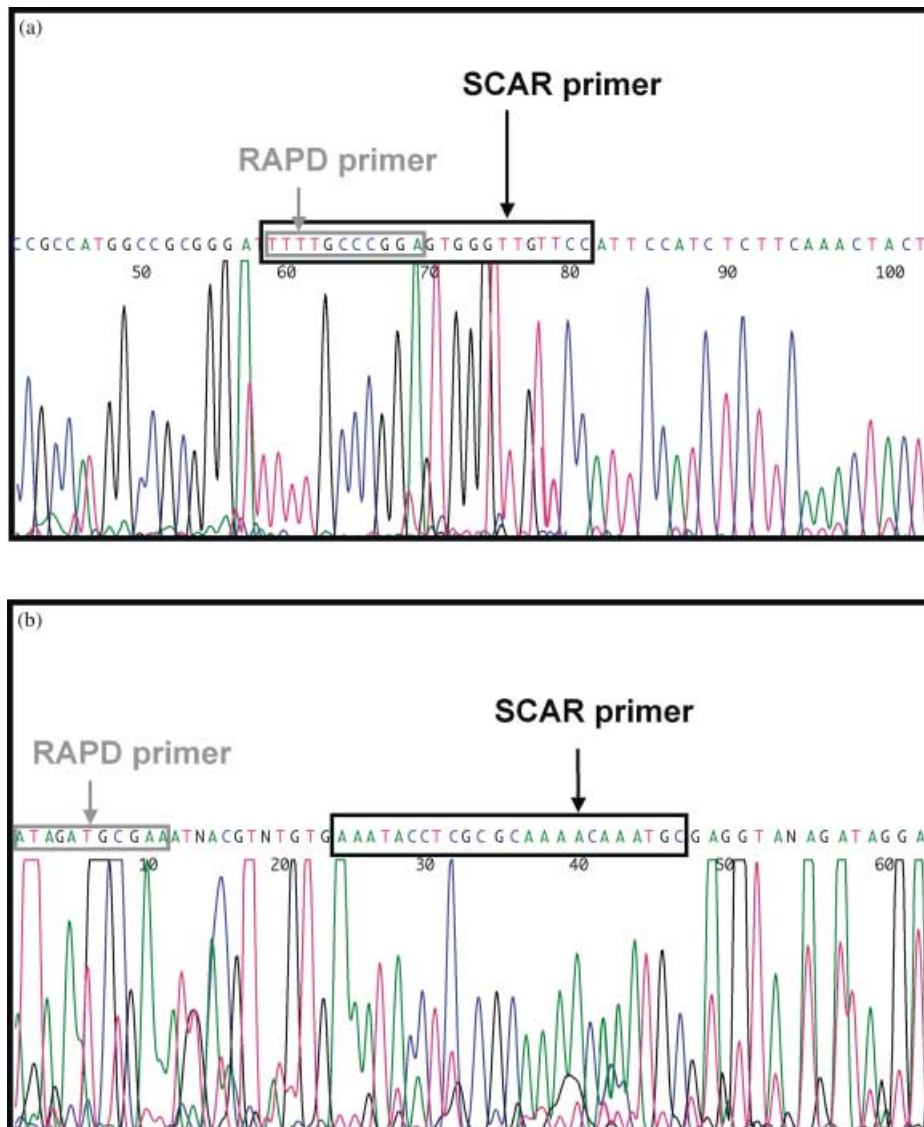


Fig. 2. SCAR primer design. a) for a cloned RAPD marker; b) for a directly sequenced RAPD marker.

poor sequencing results. Nevertheless, when SCARs are developed by cloning, the same phenomenon leads to clones containing different RAPD fragments of identical molecular weight with no information available to determine the targeted RAPD sequence. In these cases, the sequence obtained by direct sequencing, although of poor quality, can sometimes be aligned only with one of the cloned sequences, allowing the selection of the correct clone (HERNÁNDEZ et al. 1999). Thus both approaches can be complementary to overcome such a problem.

An alternative approach for direct sequencing of RAPD products is the use of a single primer for amplification (the standard RAPD technique) and the four possible sets of 3'-extended oligonucleotide primers for dye terminator cycle sequencing

(MITCHELSON et al. 1999). The cloning step is also avoided. So far, the development of SCAR markers using the obtained sequences has not been reported.

The use of wheat and barley SSR and STS primers

Wild species like *H. chilense* can benefit from marker resources developed in related Triticeae crop species such as barley and wheat. For example, a subset of SSR and STS primers developed for wheat and barley have an additional application for marker-assisted *H. chilense* introgression into wheat.

There is little information available to predict transferability of SSR or STS markers, but their ease of assay enables them to be tested empirically. As large numbers of SSR and STS markers are being

developed in wheat and barley, even a relatively low level of transferability provides a valuable marker resource (see Fig. 1c and 1d for amplification examples of STS and SSR markers). In the present case, more than 50% of wheat and barley primer pairs amplified products from *H. chilense* DNA and thus would be applicable for mapping and germplasm surveys. Some of the *H. chilense* products failed to amplify in tritordeum or the addition lines, probably because of preferential amplification of the wheat sequences, but 36% of wheat and 29% of barley markers could be used for analysis of tritordeums and derived introgression lines. These figures clearly show that wheat and barley SSRs constitute an efficient and cost effective source of molecular markers for *H. chilense* (HERNÁNDEZ et al. 2002a). About 90% of the barley STS primers tested amplified the *H. chilense* genome. About 10% of them have proven useful for genetic analysis of tritordeums and derived introgression lines (HERNÁNDEZ et al. 2002b).

Marker-assisted selection of derived introgression products

Traditionally, marker-assisted selection of basic breeding material has been accomplished using morphological or biochemical markers. The advent of DNA markers widens the possibilities of application. Marker-assisted selection of basic breeding material has been carried out, in a first approach, with the development of chromosome-specific markers for *H. chilense*, detectable in a wheat background. These markers are currently being used to obtain new addition lines of *H. chilense* accessions carrying agronomically interesting traits. The bread wheat × hexaploid tritordeum hybrid (AABBH^{ch}D) is in some varietal combinations self-fertile and seed set can always be obtained after backcrossing with either of the parents (wheat or tritordeum). In this way, tritordeum can be used as a bridge for transferring desirable traits from *H. chilense* into bread wheat (MARTÍN et al. 1998). Short-term goals are the introgression of genetically or chromosomally characterized traits. For example, high carotenoid content, interesting for durum wheat breeding, is conferred by the addition of the alpha arm of chromosome 7 H^{ch} (ALVAREZ et al. 1998) and resistance to carnal bunt is located on 4 H^{ch} (MARTÍN et al. 1998). With this aim, *H. chilense* chromosome-specific SSR, STS and SCAR markers will be used.

Additional goals are the marker-assisted selection and chromosomal characterization of tritordeum nullisomic lines. This will be carried out using wheat SSRs specific for the A and B genome chromosome arms, as well as SSRs, SCARs and STSs marking the *H. chilense* chromosome arms.

Finally, barley STSs and SSRs that are not transferable to *H. chilense* are detected in the primer screening process. In conjunction with in situ hybridisation techniques these constitute valuable markers for the marker-assisted selection of tritordeum addition and substitution lines that contain *H. vulgare* chromosomes or chromosome segments (HERNÁNDEZ et al. 2002a).

ACKNOWLEDGEMENTS

Financial support from the Spanish 'Ministerio de Ciencia y Tecnología' ('Ramón y Cajal' Program and project AGL2002-04079-C02-02 from DGI) is gratefully acknowledged.

REFERENCES

- Alvarez J, Martín A and Martín L, (1998). Chromosomal location of genes for carotenoid pigments using addition lines of *Hordeum chilense* in wheat. *Plant Breeding* 117: 287–289.
- Hernández P, Rubio MJ and Martín A, (1996). Development of RAPD markers in tritordeum and addition lines of *Hordeum chilense* in *Triticum aestivum*. *Plant Breeding* 115: 52–56.
- Hernández P, Rubio MJ and Martín A, (1995). RAPDs as molecular markers for the detection of *Hordeum chilense* chromosomes in wheat addition lines and in tritordeum. *Chromosome Res.* 3: 100.
- Hernández P, Martín A and Dorado G, (1999). Development of SCARs by direct sequencing of RAPD products: a practical tool for the introgression and marker-assisted selection of wheat. *Mol. Breeding* 5: 245–253.
- Hernández P, Dorado G, Cabrera A, Laurie DA, Snape JW and Martín A, (2002a). Rapid verification of wheat-*Hordeum* introgressions by direct staining of SCAR, STS and SSR amplicons. *Genome* 45: 198–203.
- Hernández P, Dorado G and Martín A, (2002b). Cross-species amplification of the *Hordeum chilense* genome using barley Sequence-Tagged-Sites (STSs). *Hereditas* 135: 243–246.
- Hernández P, Laurie DA, Martín A and Snape JW, (2002c). The utility of barley and wheat Simple Sequence Repeat (SSR) markers for the genetic analysis of *Hordeum chilense* and tritordeum. *Theoret. Appl. Genet.* 104: 735–739.
- Islam AKMR, Shepherd KW and Sparrow DHB, (1978). Production and characterization of wheat-barley addition lines. In: *Proc. 5th Int. Wheat Genet Symp.*, p. 365–371, New Delhi.
- Kleppe K, Ohtsuka E, Kleppe R, Molineux R and Khorrana HG, (1971). Studies in polynucleotides XCVI: repair replication of short synthetic DNAs as catalyzed by DNA polymerases. *J. Mol. Biol.* 56: 341–361.
- Martín A, Martín LM, Cabrera A, Ramirez MC, Gimenez MJ, Rubiales D, Hernández P and Ballesteros J, (1998). The potential of *Hordeum chilense* in breeding *Triticeae* species. In: *Triticeae III* (ed. AA Jaradat). Science Publishers, Enfield, NH, USA, p. 377–386.
- Martín A, Martínez-Araque C, Rubiales D and Ballesteros J, (1996). Tritordeum: triticale's new brother cereal. In:

- Triticale: today and tomorrow (eds H Guedes-Pinto, N Darvey and VP Carnide). Kluwer Academic.
- Miller TE, Reader SM and Chapman V, (1982). The addition of *Hordeum chilense* chromosomes to wheat. In: Proc. Int. Symp. Eucarpia on Induced variability in plant breeding (ed. C Broertjes). Pudoc, Wageningen, p. 79–81.
- Mitchelson KR, Drent J, Duong H and Chaparro JX, (1999). Direct sequencing of RAPD fragments using 3'-extended oligonucleotide primers and dye terminator cycle-sequencing. *Nucleic Acids Res.* 27: e28.
- Mullis KB and Faloona F, (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155: 335–350.
- Murray YHG and Thompson WF, (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321–4326.
- Panet A and Khorana HG, (1974). Studies on polynucleotides. The linkage of deoxyribopolynucleotide templates to cellulose and its use in their replication. *J. Biol. Chem.* 249: 5213–5221.
- Paran I and Michelmore R, (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoret. Appl. Genet.* 85: 985–993.
- Peil A, Schubert V, Schumann E and Weber WE, (1997). RAPDs as molecular markers for the detection of *Aegilops markgrafii* chromatin in addition and euploid introgression lines of hexaploid wheat. *Theoret. Appl. Genet.* 94: 934–940.
- Saiki RK, Bugawan TL, Horn GT, Mullis KB and Erlich HA, (1986). Analysis of enzymatically amplified b-globine and HLA-DQa DNA with allele-specific oligonucleotide probes. *Nature* 324: 163–166.
- Saiki RK, Scharf SJ, Faloona F, Mullis KB, Horn GT, Erlich HA and Arnheim N, (1985). Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350–1354.
- Vaz Patto MC, Aardse A, Buntjer J, Rubiales D, Martín A and Niks RE, (2001). Morphology and AFLP markers suggest three *Hordeum chilense* ecotypes that differ in avoidance to rust fungi. *Can. J. Bot.* 79: 204–213.
- Welsh J and McClelland M, (1991). Genomic fingerprinting with AP-PCR using pairwise combinations of primers: application to genetic mapping of the mouse. *Nucleic Acids Res.* 19: 5275–5279.