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# A high-throughput DNA extraction method for barley seed

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**Summary**

A non-destructive, quick DNA extraction method for barley seed is described. The method is simple and consists of drilling out a sample from the seed, adding sodium hydroxide, heating in a microwave oven and neutralizing with Tris-HCl. The seed DNA extract can be used directly for PCR with extra cycles added to the PCR programme compared to PCR programmes used for leaf extracts. This protocol was developed in particular for a microsatellite marker genetically linked to barley yellow mosaic virus resistance, but it can be applied to other markers of interest for barley breeding. The quick seed extraction protocol makes it possible to handle thousands of samples per day. Extraction of DNA from seed also facilitates transfer of plant material compared to the long-distance transfer of leaf samples.

## Introduction

In plant breeding selection is performed for desirable agronomic traits. Traditionally, the selection is based on phenotype, e.g. selection for disease resistance can be performed by growing plants in an infected field and assessing infection.

Genotypic selection, particularly at the DNA level, is rapidly becoming a preferred alternative, and can be exploited in Marker Assisted Selection (MAS) to identify desirable recombinants among segregating populations. The process is non-destructive and after selection the plants and the results can be returned to the breeder who then can progress material speedily in the breeding programmes.

A high-throughput MAS system requires simple and rapid DNA extraction methods. Normally, extraction of DNA is performed on leaf tissue, which requires growing plants. This is a waste of seed, glasshouse and staff resources. In our lab two DNA extraction protocols are currently used; a standard protocol (Cheung et al., 1993) that yields high quality DNA with which one person can extract 48 to 72 samples per day, and a quick extraction protocol (Dayteg et al., 1998; Tuvesson et al., 1998) that yields low quality DNA but nevertheless good enough for routine analyses. The quick DNA extraction procedure for leaf samples simply consists of treatment with sodium hydroxide (NaOH), heating (on boiling water), crushing and neutralizing with Tris-HCl, enabling one person to extract 5000 samples per day.

DNA extraction performed on seed instead of leaf tissue allows MAS to be carried out independently of the growth season, and the time and glasshouse space needed for growing the plants are saved. Most importantly, the seed can be analysed during the non-field season, selected and prepared for the next breeding cycle. Furthermore it is possible to send seed samples internationally, this is difficult for leaf samples which have to be kept on ice or lyophilised. DNA extraction performed on seed has been described using single dry seed of wheat and rice (Chungwungse et al., 1993) and seed of 12 species including wheat, rice and barley (Kang et al., 1998).

This paper describes the development of a quick DNA extraction protocol from barley seed.

## Materials and methods

*Plant material*

Seed (caryopses) of winter barley cultivars Frost, Grete, Igri, Jana (*rym4*), Misato Golden (*rym5*), Mokusekko (*rym5*) and Vixen (*Yd2*) and spring barley cultivars Alexis, Alva, Cecilia, Chevron (*Rpg1*), Coracle (*Yd2*), Ellice (*Rpg1*), Filippa, Goldie, Hanka (*Rph7*), Lina, Meltan, Mentor, Pongo, and SW 3756-99 (*Rph16*). Seed of a $F_2$ winter barley population, SW 99692, segregating for *rym5*.

*DNA extraction from seed*

A one-mm diameter drill-bit (Velleman$^R$ electric drill & engraving set. VTHD21B-DC 18V, 12–20000 rpm) was used to bore out samples from dry barley seed. While drilling, the seed were kept in place with forceps on an aluminium surface (Figure 1). *Note: caution should be taken during the drilling step*. The homemade aluminium tray was prepared using a punch and hammer to make indentations in the aluminium surface. The drill-bit and the aluminium tray can be cleaned easily between samples by action in water. After drilling, the seed was positioned in a microtitre plate and the flour positioned with a small spoon in the corresponding well of another plate for extraction: 40$\mu$l 0.15 M NaOH were added to each sample and heated in a microwave oven at 10% power of 700 W for one minute. Next, 150$\mu$l 0.03M Tris-HCl, pH 8.0, 1 mM EDTA was added and the samples were left to settle at +4 °C for at least one hour before PCR. Samples were diluted 1:10 or 1:20 with cold water before PCR. One to ten extra cycles were added to the established PCR programmes for leaf DNA.

*PCR conditions*

PCR reactions were performed on a Peltier Thermal Cycler from MJ Research. All PCR experiments took place in a total volume of 25 $\mu$1 with 2 $\mu$l seed DNA extract (diluted in water or undiluted) and 2.5 $\mu$1 10 × buffer (*10x*, 200 mM Tris-HCl, pH 8.4, 500 mM KCl, Gibco BRL) was used for all experiments. The stock solutions for PCR were: 50 mM $MgCl_2$ (Gibco BRL), 100 mM d'NTPs (Amersham Pharmacia Biotech), primer F (10 pmol/$\mu$1), primer R (10 pmol/$\mu$1) and *Taq* DNA polymerase (5 u/$\mu$l, Gibco BRL).

*Barley Yellow Mosaic Virus resistance, rym4, rym5*

The marker for barley yellow mosaic virus (BaYMV) resistance, *rym4*, *rym5*, (Graner et al., 1999), was Bmac0029 (primer sequence available from The Scottish Crop Research Institute, R Waugh). The seed DNA extract was diluted 1:10. From stock solutions 0.75 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 0.75 $\mu$l of each primer and 0.15 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 1 cycle of (13 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C), 40 cycles of (30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C), 5 min at 72 °C, 20 °C 'forever'. This is the PCR protocol established for leaf DNA with 10 additional cycles.

*Barley Yellow Dwarf Virus resistance, Yd2 (YLM)*

The marker for barley yellow dwarf virus (BYDV) resistance, *Yd2*, (YLM) was published by Paltridge et al., 1998. Undiluted seed DNA extract was used for PCR. From the stock solutions 0.75 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 1.5 $\mu$l of each primer and 0.2 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 42 cycles of (30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C), 10 °C 'forever'. This is the PCR protocol established for leaf DNA with 2 additional cycles.

*Barley Yellow Dwarf Virus resistance, Yd2 (YLPRAS)*

The PCR marker for BYDV resistance, *Yd2*, (YLPRAS) was published by Ford et al., 1998. The seed DNA extract was undiluted. From the stock solutions 2.25 $\mu$l $MgCl_2$, 0.3 $\mu$l d'NTPs, 0.6 $\mu$l of each primer and 0.1 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 39 cycles of (1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C), 10 °C 'forever'. This is the PCR protocol established for leaf DNA with 4 additional cycles.

*Leaf rust resistance, Rph16*

To the PCR mix for the marker for leaf rust resistance, *Rph16*, (MWG2133) (Ivandic et al., 1998) was added undiluted seed DNA extract. From the stock solutions 0.75 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 1.25 $\mu$l of each primer and 0.2 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 5 min at 94 °C, 37 cycles of (30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C), 5 min at 72 °C, 20 °C 'forever'. This is the PCR protocol established for leaf DNA with 2 additional cycles.

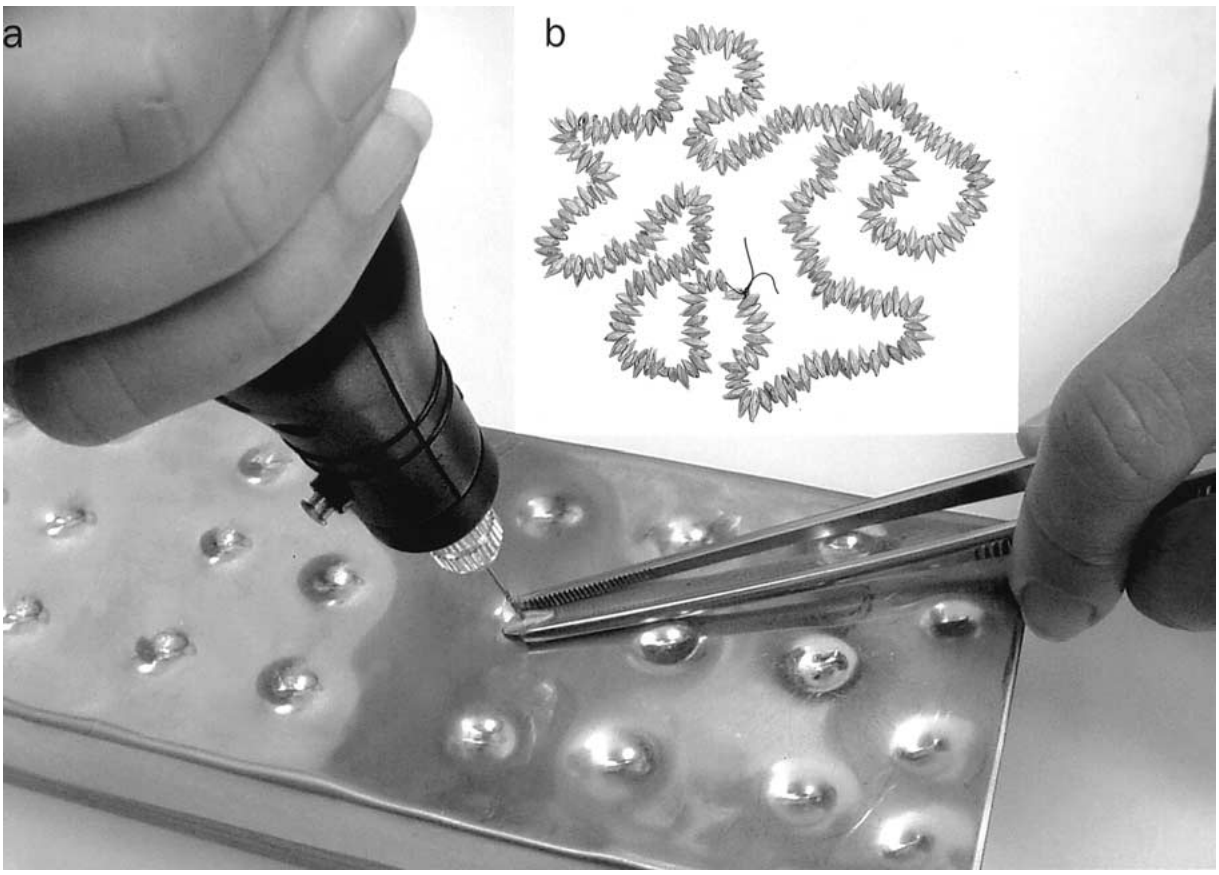


*Figure 1.* a. Samples were taken from barley seed using a one-mm drill-bit and DNA extracted from the flour. The seed can be grown on by the breeder at any suitable time. b. Necklace of barley individuals homozygous for a BaYMV marker.

*Stem rust resistance, Rpg1*

To the PCR mix used for the marker for stem rust resistance, *Rpg1*, (ABG077) (Horvath et al., 1995) was added undiluted seed DNA extract. From the stock solutions 0.75 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 1.25 $\mu$l of each primer and 0.2 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 40 cycles of (30 s at 94 °C, 45 s at 50 °C, 1 min at 72 °C), 20 °C 'forever'. This is the PCR protocol established for leaf DNA with no additional cycles.

*Leaf rust resistance, Rph7*

A marker associated with leaf rust resistance, *Rph7*, (MWG848), (Mano et al., 1999), was made available from Institut für Pflanzengenetik und Kulturpflanzenforschung, (Dr A. Graner). The seed DNA extract was diluted 1:10. From the stock solutions 1.0 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 0.75 $\mu$l of each primer and 0.125 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 5 min at 95 °C, 34 cycles of (1 min at 95 °C, 1 min at 62 °C, 2 min at 72 °C), 7 min at 72 °C, 20 °C 'forever'. This is the PCR protocol established for leaf DNA with 4 additional cycles.

*Epi-heterodendrin, Eph*

Primer sequences for the marker (Bmac0213) for epi-heterodendrin (EPH) production, *Eph,* (Swanston et al., 1999) was provided by the Scottish Crop Research Institute, R Waugh. The seed DNA extract was diluted 1:10 . From the stock solutions 0.75 $\mu$l $MgCl_2$, 0.2 $\mu$l d'NTPs, 0.75 $\mu$l of each primer and 0.15 $\mu$l *Taq* DNA polymerase were added to the PCR mix. The PCR programme was: 1 cycle of (13 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C), 34 cycles of (30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C), 5 min at 72 °C, 20 °C 'forever'. This is the PCR protocol established for leaf DNA with 4 additional cycles.

*Gel electrophoresis*

*Bmac0029, Bmac0213 and YLM:* The fragments were separated on 3.5% MetaPhor agarose gel (BioWhittaker Molecular Applications) in 1 × TBE (89 mM Tris-borate, boric acid 89 mM, 2 mM EDTA pH 8.0) Running buffer was 1 × TBE and the conditions for electrophoresis were 200 V, 250 mA for 1.3 h.

*ABG077, MWG848 and YLPRAS markers:* The fragments were separated on 1.4% standard agarose gel (Saveen) in 1 × TBE. Running buffer was 1 × TBE and the conditions for electrophoresis were 200 V, 250 mA for 1.3 h.

*MWG2133:* The DNA products were separated on 1.0% standard agarose gel (Saveen) in 1 × TBE. Running buffer was 1 × TBE and the conditions for electrophoresis were 180 V, 200 mA for 6.3 h.

The 300 ml gels contained 25 $\mu$l ethidium bromide (10 mg/ml) and the results were visualised by UV light.

## Results and discussion

Extraction experiments were carried out on dry seed of winter and spring barley varieties. The project was divided into three parts: 1) sampling, 2) extraction and 3) PCR. During development of the final extraction protocol the extracts were tested for PCR with a microsatellite marker, Bmac0029 genetically linked to BaYMV resistance (Graner et al., 1999). The final extraction protocol was verified using six PCR based markers used for MAS and the PCR procedure optimised for seed DNA extract. Finally an $F_2$ population segregating for BaYMV resistance were analysed from seed and leaf.

*Sample preparation*

To find the best way of taking a sample from the seed different methods were tried. Our aim was to find a way of extracting DNA from barley seed that is quick and simple, gives DNA that is pure enough for PCR, does not kill the seed and can be done in the microtiter (96 well) format. Cutting pieces of varying sizes from the seed creates the need for a crushing/grinding step and since barley seed tissue is very hard this cannot be easily done by hand with a pestle. Various approaches were tried in order to find a way to crush the seed fairly quickly and easily: a) crushing seed pieces of varying sizes between papers with a hammer (which is not very efficient because insufficient cell walls are broken). Also, barley seed consist mostly of starch which, when subjected to sodium hydroxide and heat, becomes a gluey pulp from which it is difficult to extract DNA. b) To avoid getting too much starch in the extraction very thin slices from the centre of the seed were cut out and subjected to the extraction procedure without crushing but this did not improve the results. c) In order to grind the material more finely seed were cut in two and the piece without the embryo abraded using sandpaper, which rendered a very fine dust that was collected with a drop of water or NaOH and a pipette. This method worked quite well but it is not practical for large scale screening. d) Another attempt at getting very finely ground samples was made by drilling a hole through the centre of a seed with a one-mm diameter drill-bit and then collecting the flour. Drilling was considered to be the best method for sampling barley seed considering both the efficiency of the extraction and the handling of the samples, and was the method chosen for the final protocol (Figure 1).

*Seed DNA extraction*

The quick DNA extraction protocol for leaf tissue currently used at our lab, in a high-throughput system, consists of the following steps: Cut leaf samples with a paper punch and placing them in a microtitre plate. Add 40 $\mu$l 0.25 M NaOH. Hold the plate on a boiling water bath for one minute, crush the samples with a 'multi pestle' and neutralize with 120 $\mu$l 0.1 M Tris-HCl. Use 1.3 $\mu$l for a 25 $\mu$l PCR reaction (Dayteg et al., 1998; Tuvesson et al., 1998).

The quick DNA extraction protocol of seed material requires some modifications due to the high starch content of cereal seed. NaOH is needed to break cell walls but the boiling step was replaced with incubation at room temperature or at 50 °C (waterbath) for varying periods of time to prevent geling. Various concentrations of NaOH were tried. We found that lowering the NaOH concentration to 0.15 M and incubating at room temperature for 50 minutes works well. The time can be shortened to 20 minutes if incubation is done at 50 °C. The Tris-HCl concentration was lowered to 0.03 M and the volume added was increased to 150 $\mu$l compared to the quick leaf extraction protocol. Adding 1mM EDTA to the Tris-HCl buffer also improved results slightly. Later in the project period we tried replacing the boiling step with heating

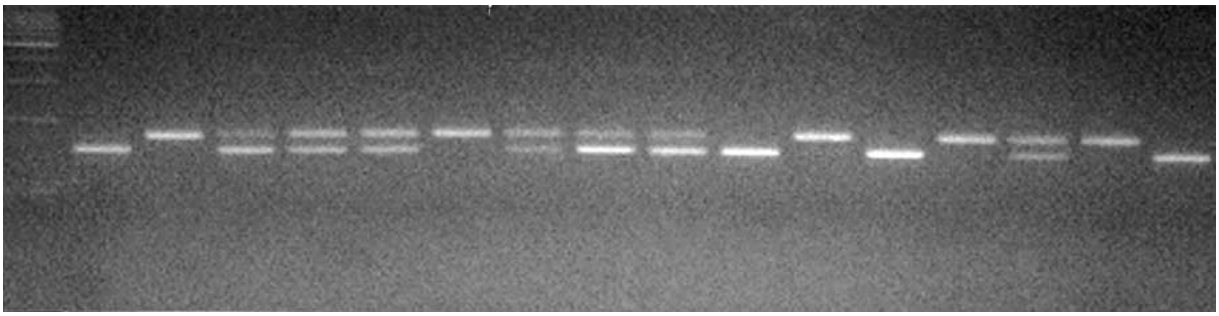

*Figure 2.* Marker assisted selection based on SSR-PCR with seed DNA extract. PCR products from Bmac0029 primer for BaYMV resistance (Graner et al., 1999). Segregating individuals from an $F_2$ population showing homozygous resistant plants (160 bp fragment), homozygous susceptible plants (176 bp) and heterozygous plants with both fragments.

in a microwave oven (Saini et al., 1999) and this gave the best results.

After extraction the samples were left to settle +4 °C for at least one hour before PCR to allow the starch, which otherwise disturbs the PCR reaction to sink to the bottom. Then the 'supernatant' can be used for PCR. The sedimentation step cannot be replaced with centrifugation since it appears that the DNA goes down with the starch and leaves the supernatant useless.

Since the extraction can be carried out in a microtitre plate the extracts can be used in an automated high-throughput MAS system.

*PCR*

During evaluation of the different sampling and extraction methods for seed, PCR was carried out with 2 $\mu$l extract for a 25 $\mu$l reaction with a microsatellite marker for resistance to BaYMV. This worked well for comparing extraction methods, but never gave very strong and reliable bands. After establishing the final quick extraction protocol the PCR protocol had to be optimized for seed extracted DNA, which is not very pure and has a very low concentration of DNA. 1) Since the extracts contain very low amounts of DNA the resulting bands are weak. Adding one to ten extra cycles to the PCR programme solved this problem. 2) To lessen the effects of PCR inhibiting substances the extract was diluted 1:10 and 1:20 in cold water after sedimentation. The undiluted extract gave low reproducibility with either of the PCR programmes, but the 1:10 dilution gave increasingly strong bands and reproducibility of the results with each added cycle.

*Verification*

The presented method was developed in particular for the marker analysis of BaYMV resistance (Graner et al., 1999) (Figure 2). Resistance to this viral disease is one of the most important characters in winter barley breeding and MAS makes possible fixation of resistance early in the breeding process.

To verify the extraction protocol and test its usefulness in MAS a final test was set up with seven different markers useful in barley breeding including the BaYMV resistance marker used for the experiments. YLM and YLPRAS are markers for *Yd2* conferring BYDV resistance (Paltridge et al., 1998; Ford et al., 1998). This viral disease is devastating to spring and winter barley in years with heavy aphid (vector) infection. Markers for resistance to rust diseases, which have fungal causal agents, also responded well to the method developed for Bmac0029: MWG848 on barley chromosome 3H (Mano et al., 1999) where an important leaf rust gene, *Rph7*, is located and ABG077 linked to the stem rust gene *Rph1* (Horvath et al., 1995) worked well with the seed DNA extract. MWG2133, linked to a new source of leaf rust resistance (*Rph16*) (Ivandic et al., 1998) responded well.The marker Bmac0213 for epi-heterodendrin (EPH) production (Swanston et al., 1999) also responded well to the developed method. EPH is a precursor for ethyl carbamate, which is an undesirable trace component in malt whisky distilling and thus important for breeding malting barley cultivars.

All markers were tested with undiluted barley seed DNA extract and with dilutions 1:10 and 1:20. The PCR reactions were run with the original PCR programme and with one, two, three, four and ten extra cycles and the results compared to results for quick extracted leaf DNA extracts (Dayteg et al., 1998;

Tuvesson et al., 1998) from the same individuals. The different markers responded differently to the various treatments and the resulting optimised seed DNA extract dilution and PCR programmes are described in the 'Materials and Methods' section. The experiments showed that the extract can be used with all the markers tested and that the seed extract and the leaf extract give similar results.

*Segregating $F_2$ population*

An issue that was further investigated is whether the seed coat, which is of maternal origin and/or the endosperm, which is triploid (2/3 maternal and 1/3 paternal) give rise to false results for the embryo. If so the technique would not be applicable to heterozygous material and therefore of limited use to plant breeders. Using the seed DNA extraction protocol 175 $F_2$ seed from a barley population segregating for BYMV resistance were analysed with Bmac0029 and the banding profiles were identical to the results obtained for leaf DNA after germination.

The successful development of a quick DNA extraction protocol for seed for use in barley breeding makes possible a much more flexible planning of marker analysis. The quick DNA extraction protocol is especially useful for laboratories which offer international DNA analysis services and which until now have been dependent upon the transfer of frozen or lyophilised leaf material.

## Acknowledgement

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## References

Cheung, W.Y., N. Hubert & B.S. Landry, 1993. A simple and rapid DNA microextraction method for plant, animal and insect suitable for RAPD and other PCR analyses. PCR Methods & Appl. 3: 69–70.

Chungwongse, J., G.B. Martin & S.D. Tanksley, 1993. Pregermination genotypic screening using PCR amplification of half-seeds. Theor Appl Genet 86: 694–698.

Dayteg, C., L. v Post, R. Öhlund & S. Tuvesson, 1998. Quick DNA extraction method for practical plant breeding programmes. Plant & Animal Genome VI. San Diego, Ca., USA, Jan 18–22, 1998, p. 39.

Ford, C.M., N.G. Paltridge, J.P. Rathjen, R.L. Moritz, R.J. Simpson & R.H. Symons, 1998. Rapid and informative assays for *Yd2*, the barley yellow dwarf virus resistance gene, based on the nucleotide sequence of a closely linked gene. Mol Breed 4: 23–31.

Graner, A., S. Streng, A. Kellermann, A. Schiemann, E. Bauer, R. Waugh, B. Pellio & F. Ordon, 1999. Molecular mapping and genetic fine-structure of the *rym5* locus encoding resistance to different strains of the Barley Yellow Mosaic Virus Complex. Theor Appl Genet 98: 285–290.

Horvath, D.P., L.S. Dahleen, J.-A. Stebbing & G. Penner, 1995. A co-dominant PCR-based marker for assisted selection of durable stem rust resistance in barley. Crop Sci 35: 1445–1450.

Ivandic, V., U. Walther & A. Graner, 1998. Molecular mapping of a new gene in wild barley conferring complete resistance to leaf rust (*Puccinia hordei* Otth). Theor Appl Genet 97: 1235–1239.

Kang, H.W., Y.G. Cho, U.H. Yoon & M.Y. Eun, 1998. A rapid DNA extraction method for RFLP and PCR analysis from single dry seed. Plant Mol Biol Reptr 16: 1–9.

Mano, Y., B.E. Sayed-Tabatabaei, A. Graner, T. Blake, F. Takaiwa, S. Oka & T. Komatsuda, 1999. Map construction of sequence-tagged sites (STSs) in barley (*Hordeum vulgare* L.). Theor Appl Genet 98: 937–946.

Paltridge, N.G., N.C. Collins, A. Bendahmane & R.H. Symons, 1998. Development of YLM, a codominant PCR marker closely linked to the *Yd2* gene for resistance to barley yellow dwarf disease. Theor Appl Genet 96: 1170–1177.

Saini, H.S., M. Shepherd & R.J. Henry, 1999. Microwave extraction of total genomic DNA from barley grains for use in PCR. J Inst Brewing 105(3): 185–190.

Swanston, S.J., W.T.B. Thomas, W. Powell, G.R. Young, P.E. Lawrence, L. Ramsay & R. Waugh, 1999. Using molecular markers to determine barleys most suitable for malt whisky distilling. Mol Breed 5: 103–109.

Tuvesson, S., L. v Post, R. Öhlund, P. Hagberg, A. Graner, S. Svitashev, M. Schehr & R. Elovsson, 1998. Molecular breeding for the BaMMV/BaYMV resistance gene *ym4* in winter barley. Plant Breed 117: 19–22.