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MAINTAINING THE EFFICIENCY OF MAS METHOD IN CEREALS WHILE REDUCING THE COSTS

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A B S T R A C T

Marker-assisted selection (MAS) is a powerful tool too rarely exploited in practical breeding applications mainly because of its prohibitive costs. A new manual protocol has been developed for DNA extraction and polymerase chain reaction (PCR) analyses which could increase the impact of this technology on the creation of new varieties. In this procedure, only the amount of DNA serving as template is extracted directly into PCR tubes. The method is reproducible (100 %) and efficient (97.9 %). The overall cost is low in term of starting lab equipment (25000 €), chemicals and consumable materials (0.33 to 0.40 € per samples) and labor (1500 sample analyses per person and per week).

Keywords: Marker-assisted selection, plant breeding, wheat, triticale.

INTRODUCTION

Marker-assisted selection (MAS) for cereal species can no more be considered as a new technology. Markers were already available in the late 1980s and since mid-1990s, numerous of PCR-based markers for interesting agronomic traits have been described in cereals. Almost 50 genes for wheat and barley can be routinely used for MAS (Miedaner and Korzun 2012). Despite the availability of this performing technology only a few released wheat varieties have been improved through MAS (Gupta *et al*., 2010). Recent technological improvements such as chip-based platforms, allow to analyze many thousands of markers per DNA sample in a short time. However this will not increase the impact of MAS in breeding programs. In practical breeding, the number of interesting markers (1 to 5) per population is low but many individuals have to be tested to keep a good level of genetic diversity. High throughput genotyping platforms improve molecular breeding (Dayteg *et al*., 2007) but only large companies can afford such kind of automated DNA extraction and PCR amplification systems. Here, we communicate a rapid and economical MAS method affordable for small breeding programs. The protocol does not require specific and expensive lab equipment or manipulation of toxic and dangerous chemicals. This method shows excellent results for wheat and triticale PCR analyses and may also be used with other plant species.

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MATERIALS AND METHODS

Green leaf tissue (2 to 4 cm length) from seedling up to flowering stage of wheat and triticale plants grown in climate chamber, greenhouse or in the field were collected in 1.2 ml plastic tubes (HydroLogix Specialty Tubes, Molecular BioProducts, FisherScientific) and placed in 96-wells boxes . The plant material could be processed directly, stored up to 3 days at 4°C or kept frozen at -80°C for several months. For DNA extraction, 0.1 ml extraction buffer (50mM Tris/HCl pH 8.0, 50 mM Na_2EDTA pH 8.0, 250 mM NaCl and 15% (w/v) sucrose) is added to each sample. The leaves are manually ground with small plastic pestle (Kimble Chase Kontes, FisherScientific) heat-adapted to fit into the tubes. Once the 96 leaf samples have been well squashed, 0.4 ml of lysis buffer (freshly made mixture consisting of 45 ml of 20 mM Tris/HCl pH 8.0, 10 mM Na_2EDTA pH 8.0 and 6 ml of 10% (w/v) of SDS) is added and samples are incubated for 30 min at 70°C under orbital shaking. To replace a centrifugation step, a 96-wells microtube plate (collection microtubes, Qiagen) in the bottom of which, 5 small holes in each well have been made with a heated needle, is carefully inserted into the tubes. The system is placed on

melting ice for 5 min and 5 µl of the filtered mixture is transferred into 0.2 ml strips of PCR tubes with an 8-channels pipette fitted with long tips (DL 10 µl tips, Gilson). The tips are kept and reused later on with the same sample. The tubes containing leaf extracts can be frozen at -80°C if other PCR analyses are desirable. DNA is precipitated with 13 µl of ethanol solution (2 ml of sodium acetate 3 M pH 5.2 mixed with 48 ml absolute ethanol), incubated for 2-5 min on melting ice and pelleted by centrifugation (Sigma 3-16 PK, vertical rotor for 192 PCR tubes, FisherScientific) at 13226 x g for at least 15 min. The supernatant is removed with the 8-channels pipette using the tips used earlier. DNA is then washed with 140 µl of 75 % (v/v) ethanol. After at least 10 min of centrifugation at 13226 x g, ethanol is removed. Following a quick spin, the last drop is sucked out by pipetting (same tip previously utilized) and the pelleted DNA is dried at room temperature for 2 to 5 min. In this extraction process, the pestle and the micotubes plates, washed and thoroughly rinsed with water, could be reused almost indefinitely.

The dry DNA pellet is diluted with 7 ml of PCR mix containing 0.2 U of HotStar Taq polymerase (Qiagen),1 X buffer and Q-Solution (proprietary Qiagen PCR additive), 1 mM of $MgCl_2$, 0.2 mM of dNTP (AppliChem), 1 mM of each primer and 0.2 mg of RNAse A (Sigma). PCR is performed on Cycler Thermo Doppio (VWR International AG) with the following conditions : 15 min at 95°C for the activation of the enzyme, then 35 cycles of 1 min at 94°C, 1 min 30 s at 55 to 65°C (depending on the primers sequences) and 2 min 30 s at 72°C. For the final extension, samples are heated for 10 min at 72°C.

The PCR products are separated on horizontal 1 to 4 % agarose (AppliChem) gels on Sub-Cell Model 192 (BioRad) using 51-wells combs (075 mm or 1.5 mm width). This type of comb allows the utilization of the 8-channels pipette to load the samples.

After DNA migration (50 to 120 Volts) in TAE buffer, the gel is stained with ethidium bromide and the DNA fragments can be scored.

RESULTS AND DISCUSSION

In our breeding group, MAS is used only when phenotypical traits are difficult or impossible to score in the field. Marker technology assists also in backcross selection, the pyramidization of genes and the choice of genitors. The protocol described here gives excellent results with any actual type of PCR based marker (SSRs, SNPs or SCARs) and is suitable for duplex or triplex-PCR amplification.

The reproducibility and the efficiency of the analysis are essential. With this method, 100% of reproducibility was achieved by testing 500 samples twice. The efficiency (table 1) has been tested on 12960 plants with several sets of primers supposed to amplify fragments in every sample and was evaluated by the presence/absence of amplified PCR fragments on agarose gels. The average failure rate is estimated at 2.1 % and probably results from a defective DNA extraction. No correlation has been observed between the failure rate and the conditions of growth or the primers used.

As the results are fully reproducible, only most promising plants containing genes or QTLs of interest are retained. The failure rate, 1% higher than published by Frey *et al*. (2004), is acceptable. Plants for which no marker could be assigned will simply be considered as worthless in subsequent breeding steps. However, the number of plant tested should be slightly increased (2.1 %) in order to keep the genetic diversity level.

One person can process approximately 1500 samples in a week (40 h) from the individual labeling and the leaves collection in the field to the data restitution to the breeder. This is over 30 % quicker when compared to other MAS methods (Frey *et al*., 2004; Aliyu *et al*., 2013).

The rapid DNA extraction protocol (Hill-Ambroz *et al*., 2002), allowing 960 extractions per day, does not provide reliable results for several markers and the failure rate increases up to 50 % under our experimental conditions.

The cost of MAS for small breeding companies is often prohibitive and the cost/benefit ratio is difficult to establish. The price of this technology could be divided into the cost for equipping a new lab and the cost of consumables and reagents. Only large companies can afford and amortize expensive fully automated lab platforms. Moreover no leaf harvesting robots have yet been invented and many working hours are needed to feed such platforms. For our MAS protocol, the lab equipment required is a centrifuge for PCR tubes, an agitated incubation oven or a water bath, a multi-channel pipette, a set of micropipettes, two thermocyclers and two large electrophoresis systems. The total cost of such equipment is lower than 25000 €.

The cost of disposable materials (Table 2) depends on the polymorphism between parental lines which affects the percentage of agarose needed. The cost of consumables per individual ranges between 0.33 and 0.40 €, which is 2 to 10 times cheaper than other MAS

methods previously described (Dreher *et al*., 2003; Frey *et al*., 2004; Bhattacharjee *et al*., 2004, Collard and Mackill 2008). The price will be considerably reduced for consumables purchased outside of Switzerland. For example, in USA, one tips pack is sold 4.3 US dollars whereas the Swiss cost is 7.98 US dollars or the same agarose powder is 25 % cheaper in Germany than in Switzerland.

At the age of genomic selection, the method presented here may seem quite archaic. No commercial kit or automated technology is required, reducing the cost of the process and allowing every breeding group to benefit from MAS advantages. In our breeding group, MAS is involved in several projects (Table 1) and so far has allowed to improve two leaf rust susceptible varieties (CH-Campala and CH-Rubli) into resistant cultivars through the introduction of Lr22a by marker assisted backcrosses.

Table 1. Efficiency of the new method estimated after 3 years (2010 to 2012) of MAS integration to breeding programs.

Species	Project	Condition of Growth	Generation	Number of DNA samples	Number of failed analysis	Primers Used	Reference
Wheat	GMO contamination	greenhouse	-	7400 plants	-	sbi143-ombob8	Beat Keller, personal communication
	Lr34	field	F7 to F9	960 lines	22 (2.3 %)	cssfr5	Lagudah *et al*. (2009)
	Tsn1	field	F7 to F9	1056 lines	-	PNL2-PNL B3	Bruce McDonald, personal communication
	Lr22a	field	F5	144 lines	1 (0.7 %)	gwm261 and wmc503	http:\\wheat.pw.usda.gov
	Lr9 + Lr24	field	F4 - F5	1056 lines	-	J09 and SCS5	Schachermayer *et al*. (1995) and http:\\wheat.pw.usda.gov
	Qfhs.ndsu-3BS	field	F2 - F3	4320 plants	103 (2.4 %)	SRST-3B1, gwm533, barc133, gwm493, Sun2-3B, barc75 and gwm389	http:\\wheat.pw.usda.gov
	Lr22a BC	climate chamber	BC1 to BC6	2448 plants	40 (1.6 %)	gwm261 and wmc503	http:\\wheat.pw.usda.gov
	Lr9 + Lr24 BC	climate chamber	BC1 to BC5	576 plants	-	J09 and SCS05	Schachermayer *et al*. (1995) and http:\\wheat.pw.usda.gov
	Pre-harvest sprouting BC	climate chamber	BC1 to BC5	1488 plants	21 (1.4 %)	ZXQ118, barc57, wmc783 and wmc118	http:\\wheat.pw.usda.gov
	Qfhs.ndsu-3BS BC	climate chamber	BC1 to BC5	288 plants	4 (1.4 %)	SRST-3B1, gwm533, barc133, gwm493, Sun2-3B, barc75 and gwm389	http:\\wheat.pw.usda.gov
Triticale	Chr.1D-3D-5D	climate chamber	BC1 to BC2 F4	9424 plants	-	cfd27, gwm52 and gwm174	http:\\wheat.pw.usda.gov
	Pre-harvest sprouting BC	climate chamber	BC1 to BC5	3312 plants	82 (2.5 %)	ZXQ118, barc57, wmc783 and wmc118	http:\\wheat.pw.usda.gov and Zhang *et al*., 2008
	total samples tested			32472 samples			

BC, backcross; "Number of failed analysis" indicates the number of samples in which the DNA could not be amplified due to DNA extraction failure.

Table 2. Costs of PCR marker analysis evaluated in euros.

	Cost for 96 Samples			
	DNA extraction	PCR reaction	Agarose gel	Total Cost
Reagent	0.37	13.44	1.19 to 7.96	15.00 to 21.77
Consumable Material	10.14	7.01		17.15
Total Cost	10.51	20.44	1.19 to 7.96	
Final Cost	-	-		32.15 to 38.92

Other promising varieties are expected from the pyramidization of pre-harvest sprouting (PHS) resistant QTLs or leaf rust resistance genes. MAS has also opened the door to projects aiming to convert Triticale to a bread making cereal.

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