Use of marker-assisted selection (MAS) for pyramiding two leaf rust resistance genes, (*Lr9* and *Lr24)* in wheat

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Abstract
Two leaf rust resistance genes, *Lr9* and *Lr24* have been pyramidised through the use of simple sequence repeats (SSR) markers. The wheat lines obtained which carry the two resistance genes are indeed resistant to the leaf rust races currently present in Switzerland and have a very good baking quality. However these lines can only be considered as a first step, since it was difficult to reach an acceptable uniformity. Furthermore, sufficient yield for commercial success could not be achieved. However they can be used as parents to cumulate other resistance genes in new elite material. This first cycle of "pyramidisation" allowed us to evaluate the cost for marker-assisted selection (MAS). MAS proved to be an efficient tool in a breeding program. It is yet necessary to integrate this benefit into the global context of yield, resistance and quality required for the release of commercially successful wheat varieties.

Key words: leaf rust, MAS, molecular markers, PCR, resistance breeding, SSR, wheat.

Introduction
Leaf rust is an important foliar disease of wheat. Growing resistant cultivars is probably the most efficient, cost-effective and environment-friendly method to control this disease (Singh *et al.*, 2004). More than 70 specific leaf rust resistance genes are known (KOMUGI, 2008). Many of them have been introgressed into wheat from wild relatives as *Lr9* from *Aegilops umbellulata* or *Lr24* from *Agropyron elongatum*. However the ability of the pathogen to adapt to new resistances by single step mutation constitutes a never-ending challenge for breeders. Frequently, the pyramiding strategy, combining several resistance genes into one cultivar, has been proposed to enhance the durability of resistances (Pedersen & Leath, 1988). Combining two or more resistance genes using classical host-parasite infection methods is highly time-consuming and needs specific virulent pathotypes that are often not available or too risky to use. Molecular biology and marker-assisted selection (MAS) offers the possibility to trace resistance genes in cultivars in an easier and more efficient way. At least 33 molecular markers linked to *Lr* resistance genes have been described at present (KOMUGI, 2008). When the project started, no virulence was found for *Lr9* or *Lr24* in the leaf rust populations in Switzerland. And, worldwide, no virulence was reported for the combination *Lr9* and *Lr24* (Schachermayr *et al*., 1995). Molecular markers and plant material for these genes were also available. Similar studies have been done by other groups in Europe (Nocente *et al*., 2007; Vida *et al*., 2005) but only little information is available on the lines and their commercial outcome. In this article, we evaluate the pyramidising of two leaf rust resistance genes by MAS in a small breeding program.

Materials and Methods
Plant material. The *Lr9* and the *Lr24* resistance gene donors ('Tranfer'/6 * "Thatcher" and 'Agent'/6 * "Thatcher") were backcrossed seven times to susceptible Swiss winter wheat cultivar "Arina" and selfed to produce the F8 generation. NILs containing *Lr9* gene were

crossed with the *Lr24* one. Three F1 populations were crossed with four advanced lines giving 765 F2 progenies on which, marker assisted selection (MAS) was used to select 194 lines containing both resistance genes. After 6 years of classical breeding, MAS was applied to confirm the presence of *Lr9* and/or *Lr24* in 30 of the F8 remaining lines.

Leaf rust and other diseases evaluation. The lines were evaluated for leaf rust and other diseases in separate nurseries using artificial infection with mixtures of isolates collected in Switzerland as describe by Michel (2001).

Homogeneity. The lines homogeneity was evaluated comparing the plant height, the spike and leaves morphology and colour of 30 head-to-row lines. Lines with insufficient uniformity were selfed one to tree more generation before testing them in yield trials.

Field trials and bread making quality. Small-plot ($7m^2$) trials with 2 replications have been carried out in 4 locations during one year. Sample collected from yield trial were used for quality parameters evaluation involving protein content and Zeleny sedimentation value (ICC-Standards methods, 1999).

DNA extraction. Genomic DNA from the 765 F2 populations was isolated from leaves tissue according to Lagudah and Appels (1991). For the 30 advanced lines, the DNA was extracted with a quick an efficient method. Two young leaves were grown in 2 ml of extraction buffer (Tris-HCl 50 mM pH 8, EDTA 50 mM ph8, sucrose 15 % (w/v), NaCl 250 mM). After centrifugation (5 min at 6'000 x g), the supernatant was removed and the pellet, suspended in 340 µl of Tris-HCl 20 mM pH8, EDTA 10 mM pH8 and SDS 1.2 % (w/v), was incubated for 15 min at 70 °C. Then 150 µl of 7,5 M ammonium acetate were added, the mixture incubated on ice for 30 min and centrifuged at 16'000 x g for 15 min. The DNA from the supernatant was concentrated by ethanol precipitation, washed with 75 % (v/v) ethanol and dissolved in TE at a concentration of 25 ng/µl.

PCR amplification. Polymerase chain reaction (PCR) was performed in 10 µl volumes with 25 ng of template, Qiagen PCR buffer and Q-solution as recommended by the manufacturer, 0.2 mM dNTPs, 1 µM of each primer, 1.5 mM $MgCl_2$ and 0.35 U HotStar Taq DNA polymerase (Qiagen). Amplifications were performed in an Hybaid PX2 thermocycler programmed at 95°C for 15 min, followed by 35 cycles at 94°C for 1 min, at 64 °C for 1 min 30 sec and at 72°C for 2 min 30 sec. The extension of amplified fragment was achieved at 72°C for 10 min. The sequences of the specific primers for *Lr9* and *Lr24* are shown in table 1.

Table 1. Sequence of primers for STS locus linked to the *Lr9* and *Lr24* resistance genes

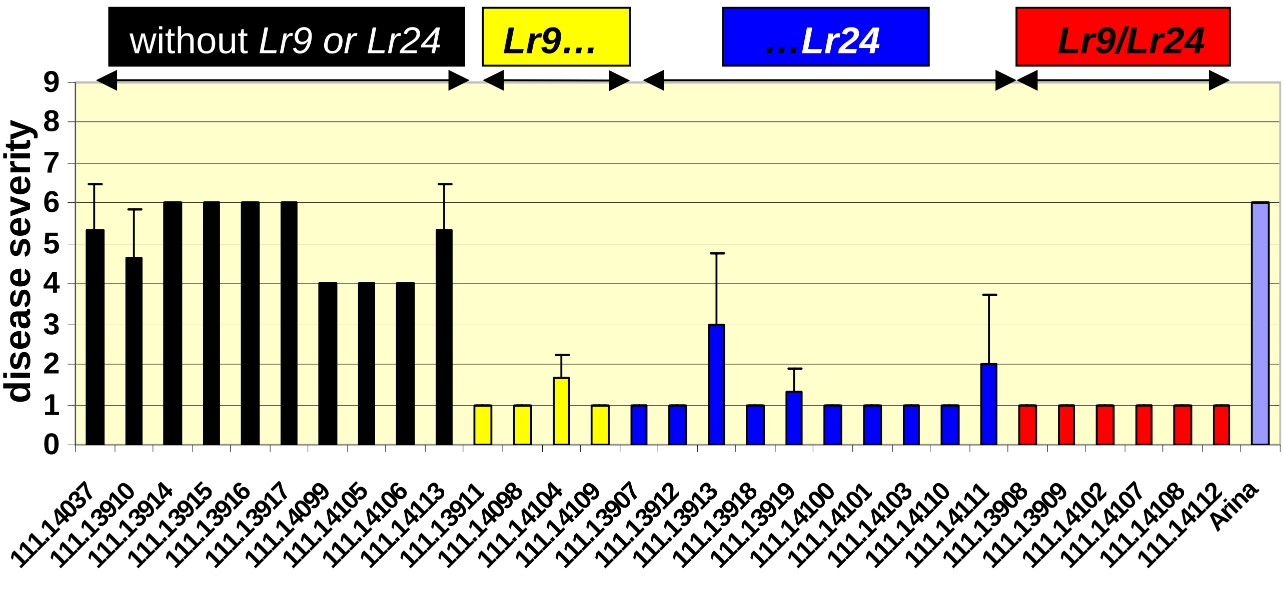
Gene	Name	Primer sequence (5' - 3')	Reference
Lr9	J13	F – CCA CAC TAC CCC AAA GAG ACG	Schachermayr *et al.*, 1994
		R – TCC TTT TAT TCC GCA CGC CGG	
	SCS5	F – TGC GCC CTT CAA AGG AAG	Gupta *et al.*, 2005
		R - TGC GCC CTT CTG AAC TGT AT	
Lr24	J09	F – TCT AGT CTG TAC ATG GGG GC	Schachermayr *et al.*, 1995
		R - TGG CAC ATG AAC TCC ATA CG	
	H05	F – AGT CGT CCC CGA AGA CCC GCT GGA	Dedryver *et al.*, 1996
		R - TCG TCC CCT GAT GCC ATG TAA TGT	

Electrophoresis. Amplification products were separated by electrophoresis on 1.5 % agarose gel in 1 X TAE buffer at 100 V for 3 h and visualized under UV light after ethidium bromide staining.

Results and Discussion

Homogeneity. At F7, only 14.3% of the 98 F7 “Lr9/Lr24 lines” reached a sufficient uniformity to be tested in yield trials and to start the maintenance breeding compared to 20.6% for the “normal” lines.

Leaf rust markers and disease resistance. The MAS at F2 with dominant PCR-based markers discard the plants without *Lr* genes but cannot sort heterozygous from homozygous plants. Lines without the genes continue to appear after self-pollination of heterozygous plants. The second PCR was performed with accurate concentration of DNA isolated from 6 to 10 plants. For the heterozygous lines, the band intensity was lower than the one obtained with homozygous lines. The heterozygosity of these lines was confirmed by analysing 6 plants separately. For the 30 lines tested in yield trials, 6 lines have markers for *Lr9* and *Lr24*, 10 lines only for *Lr24*, 4 lines only for *Lr9* and 10 lines have no markers for neither resistance genes. In fact, the susceptibility to leaf rust confirmed the role of *Lr9* and *Lr24* in the resistance response. Even if the virulence *Lr9* is now frequently observed in Switzerland (F. Mascher, data not published), lines possessing *Lr9* show few or no symptoms. On the other hand, some lines possessing *Lr24* markers display low symptoms even if virulence *Lr24* has not been reported in Switzerland. The few lines with both *Lr* markers have absolutely no symptoms (Fig.1). Arguably the pyramidisation of both resistance genes is feasible and the results obtained are efficient but the durability of the resistance has still to be proved. The mean resistance of these lines against other important diseases is very good for stripe rust (note 2.0), septoria nodorum blotch (index leaf 79, index spike 85) and good for septoria tritici blotch (index 91), powdery mildew (note 2.4) and fusarium head scab (3.2).



Yield trial. The 30 lines mean yield is only 90% of the usual standards cultivars hence insufficient for a commercial cultivar. Even if the number of lines is certainly insufficient to compare with confidence the yield between the lines with or without *Lr* markers or between the lines from different crosses we observe that the relative yield for the 6 lines with *Lr9* and *Lr24* markers is 94.2% (86.5% to 100.9%) compared to 88.8% (77.7% to 97.4%) for the 10 lines without *Lr* markers. The 8 lines issued from the cross with the best parent have a 94.1% relative mean yield compared to 80.8% for the 4 lines issued from the lowest yielding parent.

Bread making quality. The 30 lines Zeleny mean value (60.2ml, 43.7ml to 73.5ml) and the protein content mean values (13.5%, 11.7% to 15.0%) indicate a good to very good bread making quality for the lines compared with the results for "Arina" (Zeleny 52.3ml, protein content 13.8%) and "Runal" (Zeleny 65.2ml, protein content 14.3%), respectively good and very good bread making quality standards.
Cost. If we don't include costs for markers development and field trials cost (sowing, treatments, sampling), we need now, in our conditions, 1 person, two weeks and a cost of 515.- € for analyzing 1000 samples.

Conclusions

The lines herein obtained, even though displaying good resistances and excellent bread making quality, had low yield and more difficulties to reach uniformity. They could not be developed as commercial cultivars and are used as genitors. The low yield and low uniformity might be unwanted effects of *Lr* genes and of "drag genes" unintentionally introduced from the wild relative. This is especially valid for the *Lr24* donor where a large segment has been translocated (Schachermayr *et al*., 1995). It might be also caused by the genetic value of the advanced lines used as genitors in this experiment. The number of lines tested here is too small to have clear evidences for one or other hypothesis. Some of the best lines have been crossed with more yielding lines and with lines with other *Lr* genes especially adult plant resistance genes to combine different kind of resistance. The MAS was effective for pyramidising two resistance genes but the investment was important just for one of the six important diseases we breed for. Even if the cost of MAS has dropped dramatically during the last decade, it is still a challenge to find the best way to use it in a breeding program with the aim of producing cultivars not only resistant against one single disease but aiming at an optimal combination between yield, resistance and quality.

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