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USING SSR MARKERS FOR AUTHENTICATION OF SEED STOCKS IN WINTER OILSEED RAPE (WOSR)

Document prepared by experts from the United Kingdom

SUMMARY

1. Currently over 100 extra plots are grown in the DUS Winter Oilseed Rape (WOSR) trials each year for authentication of different seed stocks. Seed samples for Value for Cultivation and Use (VCU) need to be authenticated against the DUS seed stock, replacement seed stocks of samples that have very little seed left have to be checked against the original seed and varieties ordered from other countries for the reference collection have one sample from the relevant testing authority and another from the breeder, which have to be authenticated against each other. The reason for this is because the DUS seed sample is classed as the definitive sample and therefore all new samples of the same variety must be shown to be true to the variety. These authentication plots are grown side by side and assessed at two or more growth stages to check whether they are the same variety. No other recording of these plots is carried out. Authentication is part of the quality assurance and traceability of the system in the United Kingdom.

2. In cereals DUS, a system has already been developed and implemented which uses electrophoresis (EP) to authenticate VCU samples and saves growing on 240-245 plots per year for barley, wheat and oats.

3. For those varieties which are shown not to match by EP the system has the advantage of allowing samples to be tested early enough to enable seed to be grown on in the field for a phenotypic assessment.

4. A similar approach using molecular markers to authenticate seed stocks against the definitive DUS stock was explored for use in oilseed rape within this project.

BACKGROUND AND PURPOSE

5. A previous project carried out at NIAB and funded by Fera tested whether SSR markers could be used reliably for authentication of VCU and replacement seed stocks, thereby replacing the necessity for field plots. The conclusions of the project outlined in the final report were that the SSR markers could differentiate between varieties if the peak patterns were visually assessed rather than scored and that multiplexing of primers was successful for two or three primer pairs. However, there were some discrepancies in the data which could be attributed to the fact that part of the work had been carried out on an old ABI machine and the remainder on a new piece of equipment and therefore the data were not considered to be sufficiently clear and robust to use for routine testing. The cost of using SSRs for authenticating seed stocks was also not low enough to produce any savings within the project, even if the data had been robust enough to use, therefore the report for the previous project proposed testing whether more than three primers could be multiplexed in order to reduce the cost of an assay.

Objectives

- To test whether the lengthy field drilled tests could be replaced with a laboratory test that would reduce the time taken to report authentication results.
- To produce a molecular test that is cheaper than the field test and more efficient in time and overall use of resources and early identification of potential problems in National List testing.
- Use the most useful set of SSR markers from the previous project to analyze a new set of varieties (DUS and VCU seed stocks) with the new ABI analyzer.
- Compare field results with molecular results.
- Multiplex markers to reduce the cost of the molecular test.

ANALYSIS AND RESULTS

Sample selection

6. DUS samples and their corresponding VCU sample in year two of DUS testing in 2009/10 were used to assess the markers (see Annex 1 for the full list of varieties).

DNA Preparation:

7. 40 to 50 seeds of each variety were germinated on moist filter paper in the dark and harvested once the cotyledons had emerged from the testa and the seedlings were large enough to handle. The seedlings were cut from the roots, and 30 seedlings collected in a bulk to represent each variety. The dried seedlings were extracted using Qiagen DNeasy 96 Plant extraction kits in accordance with the manufacturer’s instructions.

DNA Amplification**:**

8. PCR reactions were prepared with 1 µl DNA template (nominally 10 ng), 1 µl x10 PCR buffer, 1 µl 25 mM MgCl2,1 µl 5 mM primer pairs, 0.1 µl 20 mM dNTP, 0.1 µl 5U/µl TAq polymerase and water to 10 µl.

Use of Markers**:**

9. The fluorescently labeled primers were supplied by Sigma Aldrich. All fragments were amplified using the following PCR cycling conditions: 92ºC for 120 seconds, followed by 35 cycles of 92ºC for 30 seconds, then 55ºC for 30 seconds, then 72ºC for 60 seconds followed by 72ºC for 600 seconds. Fragments were visualized using an ABI 3130 Genetic Analyzer. Data were analyzed on software Genemapper v 3.7.

Testing of suitable markers

10. 12 markers were selected as showing the greatest polymorphism between varieties (Table 1) and were used to assess the samples listed in Annex 1.

Table 1 – SSR markers selected

Marker No.	Marker	5' primer sequence	3' primer sequence	Chromosome	No. of Alleles *
M1	Ra2-E03	AGGTAGGCCCATCTCTCTCC	CCAAAACTTGCTCAAAACCC	10	3
M2	BN12A	GCCGTTCTAGGGTTTGTGGGA	GAGGAAGTGAGAGCGGGAAATCA	13	2
M5	LS107	GTTAAGTGTGGCGTTAGAGG	CCTTGGTACATGCCACTGAA	Unknown	3
M6	MB5	AACATCTTTTTGCGTGATAT	AATAGCATTGAAGCCTTAC	Unknown	2
M8	Na10-H03	GAGCTGGCTCATTCAACTCC	CACAATTTCTCAGACAAAACGG	Unknown	2
M9	Na10-E02	TCGCGCATGTAATCAAAATC	TGTGACGCATCCGATCATAC	5	3
M14	Na14-H11	GGATGTTTTCACAGACCCTG	CTTTGCAGGTATGAACACGC	Unknown	4
M16	Ol10-B01	CCTCTTCAGTCGAGGTCTGG	AATTTGGAAACAGAGTCGCC	17	4
M17	Ol10-BF11	TTTGGAACGTCCGTAGAAGG	CAGCTGACTTCGAAAGGTCC	11	2
M21	Ol12-F02	GGCCCATTGATATGGAGATG	CATTTCTCAATGATGAATAGT	9	4
M22	Ol13-C12	AGAGGCCAACAAAGAACACC	GAAGCAGCACCAGTGACAAG	13	3
M25	Ra2-A11	GACCTATTTTAATATGCTGTTTTACG	ACCTCACCGGAGAGAAATCC	9	4

* based on analysis of 410 varieties in the CPVO project “Management of Winter Oilseed Reference Collections”

Results of marker assessment of DUS and VCU seed stocks

11. The seed samples used were all found to be authentic by the field assessment. The data from the molecular analyses were initially collated into an Excel spreadsheet, containing the scored band molecular weights (“bins”) of detected bands (see Table 2 for example of the data format). Only bands of more than a third of the intensity of the major band peak for each marker were scored (relative thresholding). The earlier project carried out at NIAB showed that scoring of the peaks produced more differences between DUS and VCU samples than simply comparing the traces by eye. Within this current project we again compared scoring with direct comparison of trace patterns. Markers 3, 8, 10, 14 and 21 did not work very

successfully the first time they were run and were therefore repeated. Marker 3 proved to be monomorphic for the set of varieties used so is of little use for distinguishing varieties. Markers 8, 10, 14 and 21 showed improved results for the repeat run.

12. Of the DUS and VCU seed stocks, Table 3 shows that using the scoring method, 14 of the 33 varieties tested showed complete matches. Twelve varieties showed a difference at one allele and five showed a difference at two alleles. One variety (2240) showed a difference at three alleles and one variety (2180) showed a difference at five alleles. When the traces were compared by eye and no scoring threshold was used, the results were much better with far fewer differences between the two seed stocks. Comparing the traces there were 26 complete matches, five varieties with a difference at one allele, one variety (2227) with a difference at two alleles and one variety (2180) with a difference at three alleles. For variety 2227, markers 16 and 25 didn't work well for the VCU sample which may explain the discrepancies seen between the two seed stocks.

13. With the new set of varieties used within this current project, there were two pairs of varieties that were indistinguishable (2209 could not be distinguished from 20206 and 2196 could not be distinguished from 2215). 2196 and 2215 are interestingly from the same breeder and therefore the breeding material is likely to be similar.

Table 2 – An example of the data format. The highlighted areas show varieties which cannot be uniquely identified

		M1		M2				M5				M6		M8		M9			M16			M17	
	Samp	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele 6			Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
1608	1		30	10	20					30		10		10		10		30			40	10	
1608	2		30	10	20					30		10		10		10		30			40	10	
2019	3	20		10	20					30			20	10		10		30			40	10	
2019	4	20		10	20					30			20	10		10		30			40	10	
20206	5		30	10	20					30		10		10				30			40	10	
20206	6		30	10	20					30		10		10				30			40	10	
2209	43		30	10	20					30		10		10				30			40	10	
2209	44		30	10	20					30		10		10				30			40	10	
2163	7	20		10	20			15		30		10		10				30			40	10	
2163	8	20		10	20			15		30		10		10				30			40	10	
2165	9	20	30	10	20			15		30		10			20			30	20		40	10	
2165	10	20	30	10	20			15		30		10			20			30			40	10	
2166	11	20		10	20					30	40	10			20	10		30			40	10	20
2166	12	20		10	20					30	40	10			20			30			40	10	
2174	13	20		10	20					30		10			20			30		30	40	10	
2174	14	20		10	20					30		10			20	10		30		30	40	10	
2175	15	20		10	20					30		10		10				30			40	10	
2175	16	20		10	20					30		10		10				30			40	10	
2180	17	20	30	10	20			15		30		10			20			30		30	40	10	
2180	18	20		10	20					30		10			20	10		30		30	40	10	
2232	59	20		10	20					30		10			20			30		30	40	10	
2232	60	20		10	20					30		10			20			30		30	40	10	
2187	19		30	10			30			30			20		20			30		30		10	
2187	20		30	10			30			30			20		20			30		30		10	
2188	21	20		10	20					30		10		10				30			40	10	
2188	22	20		10	20					30		10			20			30			40	10	
2190	23	20		10	20					30	40	10			20			30			40	10	20

Table 3 – Numbers of differences in alleles between DUS and VCU samples

VARIETY	NUMBER OF ALLELES WHERE DIFFERENT USING SCORING	MARKERS WHERE DIFFERENCES SEEN	NUMBER OF ALLELES WHERE DIFFERENT BY COMPARING TRACES	COMMENTS
1608	Complete match	None	Complete match	
2019	Complete match	None	Complete match	
20206	Complete match	None	Complete match	
2209	Complete match	None	Complete match	
2163	1	M8	Complete match	M8 didn't work well
2165	1	M16	Complete match	Trace shows complete match
2166	2	M9, M17	Complete match	Trace shows complete match
2174	Complete match	None	Complete match	
2175	1	M8	1	Trace shows 1 allele difference
2180	5	M1, M5, M9, M22, M25	3	Trace shows 3 differences (M5, M9, M22)
2232	Complete match	None	Complete match	
2187	Complete match	None	Complete match	
2188	1	M8	1	Trace shows 1 allele difference
2190	Complete match	None	Complete match	
2191	1	M9	Complete match	Trace shows complete match
2192	1	M1	Complete match	Trace shows complete match
2194	Complete match	None	Complete match	
2196	1	M8	Complete match	Trace shows complete match
2215	1	M5	Complete match	Trace shows complete match
2197	1	M5	Complete match	Trace shows complete match
2198	Complete match	None	Complete match	
2199	2	M2, M8	Complete match	Trace shows complete match
2201	2	M6, M16	1	Trace shows 1 allele difference (M6)
2202	2	M8, M9	1	Trace shows 1 allele difference (M8)
2209	Complete match	None	Complete match	
2210	1	M5	Complete match	Trace shows complete match
2216	Complete match	None	Complete match	
2220	1	M5	Complete match	Trace shows complete match
2221	Complete match	None	Complete match	
2227	2	M16, M25	2	VCU trace didn't work well for both markers
2231	Complete match	None	Complete match	
2240	3	M8, M9, M25	1	Trace shows 1 allele difference (M8)
2247	1	M5	Complete match	Trace shows complete match

Multiplexing

14. Multiplex PCR allows simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. In this project several markers (primer pairs) were used to generate a genetic profile of the variety, and initially each primer pair was analyzed in a separate reaction. Individual analysis of the markers is time consuming and costly, therefore multiplexing of four primer pairs at a time was assessed to make the assay more efficient. The first step in designing a multiplex PCR is choosing primer pairs that can be combined for optimal amplification of all loci under the same conditions. Some adjustments in cycling conditions and primer concentrations were made for the combined primer pairs. Once optimization was complete, results of the multiplexed PCR matched those of the PCR using individual primer pairs.

CONCLUSIONS

15. The results of the molecular analyses show that not all varieties have exactly the same profile between different seed samples, despite matching when assessed by phenotype in the field. Differences seen between the duplicate DUS samples show that there is an inherent degree of heterogeneity within oilseed rape which should be taken into consideration when assessing whether or not two samples are considered to be the same variety. When thresholding was applied, there were more differences between DUS and VCU samples, and fewer differences between varieties than when peak heights were scored directly from the traces without thresholding.

16. The relative response for each allele within a profile will depend on the proportion of individuals within the bulked sample possessing that allele. The relative response may also be affected by the efficiency of PCR for the fragments being amplified. The size of the fragment being amplified and presence of competing fragments have an effect on PCR efficiency, therefore the peak sizes for each allele may vary from sample to sample within the same variety. This could result in alleles being called in one sample but when the relative response is weak in another sample they are not called and given a “0” in the data set. This, and the heterogeneous nature of oilseed rape, would explain why such differences were seen between the DUS and VCU samples.

17. Multiplexing of the PCR primer pairs was very successful and allowed four primer pairs to be used together in the same reaction, increasing the efficiency and reducing the overall cost of each sample.

18. The molecular test at this stage is likely to be just as accurate, if not more so, than the field test. The field test can be affected by environmental factors and because it is a visual assessment can be subjective. Even in side by side plots, one plot could have less even germination or fewer plants than its neighboring plot of the same variety. All these factors can have an effect on the appearance and growth stage of the plants. The molecular test is not influenced by the environment and it is hoped that two samples of the same variety from different sources would have matching profiles. Taking into account the heterogeneous nature of oilseed rape, the small number of differences seen between some of the duplicate samples is not surprising.

19. This test could be applied to variety identification. All the markers used within this project are in the public domain so there is no restriction on their use. Although the results are not 100% predictive of specific varieties, it is possible to say that a profile matches that of a reference sample used to ascertain whether a variety is what it is supposed to be. It should also be possible to show that a variety is not something that it should be if the profiles do not match. This is the approach currently used in electrophoresis variety identification where a profile is matched to a database and it is possible to allocate that profile to a group of putative varieties. It is possible to say therefore that a profile does or does not match that of the reference sample representative of the variety.

20. IMPLEMENTATION

21. The test will be implemented in autumn 2011 and will be analyzed as soon as possible after receipt although may not be practical to analyze all samples and obtain results before drilling as there is only a short time span of three to four weeks between the receipt of seed samples and drilling. Duplicate samples with up to two allele differences will be considered to be the same. Any duplicate seed samples showing a difference in alleles of more than two alleles would be sown in the field for phenotypic verification.

22. Authentications are currently sown in the autumn trial because the quality of the plots produced tends to be better due to the time of sowing. Due to the short time span between the receipt of the seed and drilling there is not be sufficient time to carry out the molecular analysis in time for the autumn drilling. If this is the case, any DUS and VCU seed samples that do not match would be sown in the spring for a phenotypic assessment.

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